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Carbon isotope effects associated with mixed-acid fermentation of saccharides by *Clostridium papyrosolvens*

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Abstract

In anoxic environments, microbial fermentation is the first metabolic process in the path of organic matter degradation. Since little is known about carbon isotope fractionation during microbial fermentation, we studied mixed-acid fermentation of different saccharides (glucose, cellobiose, and cellulose) in *Clostridium papyrosolvens*. The bacterium was grown anaerobically in batch under different growth conditions, both in pure culture and in co-culture with *Methanobacterium bryantii* utilizing H_2/CO_2 or *Methanospirillum hungatei* utilizing both H_2/CO_2 and formate. Fermentation products were acetate, lactate, ethanol, formate, H_2, and CO_2 (and CH_4 in methanogenic co-culture), with acetate becoming dominant at low H_2 partial pressures. After complete conversion of the saccharides, acetate was ^{13}C-enriched ($\alpha_{sacc/ac} = 0.991–0.997$), whereas lactate ($\alpha_{sacc/lac} = 1.001–1.006$), ethanol ($\alpha_{sacc/etoh} = 1.007–1.013$), and formate ($\alpha_{sacc/form} = 1.007–1.011$) were ^{13}C-depleted. The total inorganic carbon produced was only slightly enriched in ^{13}C, but was more enriched, when formate was produced in large amounts, as $^{12}CO_2$ was preferentially converted with H_2 to formate. During biomass formation, ^{12}C was slightly preferred ($\alpha_{sacc/biom} \approx 1.002$). The observations in batch culture were confirmed in glucose-limited chemostat culture at growth rates of 0.02–0.15 h^{-1} at both low and high hydrogen partial pressures. Our experiments showed that the carbon flow at metabolic branch points in the fermentation path governed carbon isotope fractionation to the accumulated products. During production of pyruvate, C isotopes were not fractionated when using cellulose, but were fractionated to different extents depending on growth conditions when using cellobiose or glucose. At the first catabolic branch point (pyruvate), the produced lactate was depleted in ^{13}C, whereas the alternative product acetyl-CoA was ^{13}C enriched. At the second branch point (acetyl-CoA), the ethanol formed was 15.6–18.6‰ depleted in ^{13}C compared to the alternative product acetate. At low hydrogen partial pressures, as normally observed under environmental conditions, fermentation of saccharides should mainly result in the production of acetate that is only slightly enriched in ^{13}C (<3‰).

1. Introduction

In the anaerobic degradation of organic matter, low-molecular-weight organic compounds are important intermediates (Ward and Winfrey, 1985; Zinder, 1993). They are products of fermentation and serve as substrates for the terminal mineralization of carbon to CO_2 and CH_4. Acetate, which in nature is usually present at only very low concentrations, plays a key role as substrate for methanogenesis, iron reduction and sulfate reduction (Ward and Winfrey, 1985; Lovley, 1993; Zinder, 1993). Primary fermenting microorganisms seem to be the major producers of acetate (Chidthaisong et al., 1999), whereas secondary fermenting (Krylova et al., 1997) and chemolithotrophic homoacetogenic bacteria (Lovley and Klug, 1983) produce much smaller amounts. In anaerobic soils and sediments, analysis of stable carbon isotopes has been used for tracing and quantifying carbon flow (Sugimoto and Wada, 1993; Hornibrook et al., 2000). The extent, to which the lighter isotope is preferred, is characteristic for distinct biogeochemical processes. With knowledge of the fractionation factors of the individual processes, it is possible to determine the dominant processes, simply by measuring the iso-

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topic composition of a few key compounds. Fractionation factors of homoacetogenesis (Gelwicks et al., 1989; Preuss et al., 1989), acetoclastic methanogenesis (Krzycki et al., 1987; Gelwicks et al., 1994) and hydrogenotrophic methanogenesis (Balabane et al., 1987; Botz et al., 1996) have been determined in pure microbial culture. However, for the calculation of the fraction of methane produced by acetoclastic methanogenesis, the $\delta^{13}C$ of acetate needs to be known. Since acetate usually cannot be measured directly due to its low steady-state concentration under natural conditions, the fractionation associated with acetate production by primary fermentation is an important information.

During fermentation carbon atoms are channeled through a metabolic network with branch points (e.g., pyruvate, acetyl-CoA), at which the carbon flow is divided into different branches (Fig. 1). At these branch points, one reactant is consumed by two or more competing reactions, each reaction having its own kinetic isotope effect. Generally $^{12}k/^{13}k > 1$, so that in linear reactions the product is always depleted in ^{13}C or has the same isotope ratio as the initial reactant, when the reaction is run to completion. At a branch point, on the other hand, the carbon isotope flow is divided and ^{12}C preferentially flows into the product generated by the reaction with the larger fractionation factor (α). Because of conservation of mass the other product must be enriched in ^{13}C. If a substrate is quantitatively consumed, linear isotope effects are not expressed and the isotope ratios of the products are only influenced by the fractionation at the branch points. Hence, quantitative substrate conversion allows examination of the isotope effects expressed at the branch points of the saccharide fermentation path. In addition, difficulties linked to intramolecular carbon specific fractionation of incomplete reactions are avoided. Such difficulties emerge for example

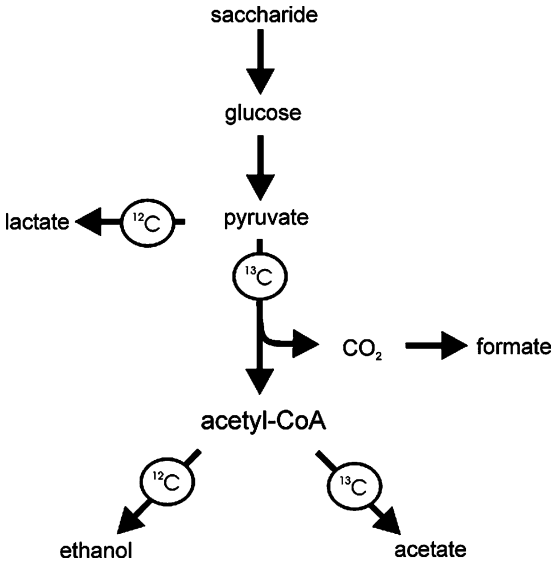

Fig. 1. Catabolic carbon flow during fermentation of saccharides by *C. papyrosolvens*. The isotope flow that was determined in this study is also included. At branch points (pyruvate and acetyl-CoA) the reactions with a stronger preference of ^{12}C are indicated by ^{12}C on the arrow; ^{13}C on the arrow indicates a less pronounced ^{12}C preference.

during the incomplete oxidation of pyruvate to acetyl-CoA by pyruvate dehydrogenase, where the ^{13}C depletion of acetyl-CoA is concentrated in the carbonyl carbon atom while only a minor effect is seen in the methyl carbon (DeNiro and Epstein, 1977).

Nevertheless, the intramolecular isotopic composition of the saccharides is important. Early studies assumed homogeneity (DeNiro and Epstein, 1977; Blair et al., 1985), but Rossmann et al. (1991) found that glucose from both maize and beet sugar had a similar intramolecular distribution of ^{13}C that was non-statistical. Carbon positions 3 and 4 of glucose were found being enriched in ^{13}C, whereas the other positions were depleted relative to the average $\delta^{13}C$ of the glucose. In the Emden–Meyerhoff–Parnas (EMP) pathway, carbon positions 3 and 4 of glucose form the carboxyl group of pyruvate. Since pyruvate is a branch point, where both lactate and acetyl-CoA + CO_2 can be formed, knowledge of the positional isotope distribution is helpful for interpretation of carbon isotope fractionation.

Knowledge of the fractionation factors involved in the formation of acetate and other fermentation products and their dependency on growth parameters is a prerequisite for modeling the later reactions in the mineralization of organic matter. So far, carbon stable isotope fractionation during saccharide fermentation has been studied only once using *Escherichia coli* (Blair et al., 1985). Therein, *E. coli* fermented glucose under aerobic conditions, so that the thus obtained fractionation factors cannot be applied reliably to the carbon flow in anaerobic systems. In addition, *E. coli* is an intestinal bacterium, which plays no role in outdoor environments. Therefore, we chose the bacterium *Clostridium papyrosolvens*, which is cellulolytic and was first isolated from an anaerobic estuarine sediment (Madden et al., 1982), later from freshwater swamps (Leschine and Canale-Parola, 1983; Pohlschröder et al., 1994), and from rice field soil (Chin et al., 1998). *C. papyrosolvens* ferments saccharides in a mixed-acid fermentation via the EMP-pathway producing acetate, CO_2, ethanol, formate, lactate, and H_2 (Fig. 1). In the present study, we investigated the stable carbon isotope fractionation associated with the fermentation of different saccharides (glucose, cellobiose, and cellulose) under batch and continuous growth conditions and tested the effect of environmental conditions like temperature, substrate concentration, and hydrogen partial pressure.

2. Materials and methods

2.1. Cultures and growth conditions

Clostridium papyrosolvens (DSM 2782), *Methanobacterium bryantii* (DSM 863), and *Methanospirillum hungatei* (DSM 864) were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The microorganisms were grown in phosphate-buffered mineral medium under N_2. The composition was (in g/L unless otherwise indicated): KH_2PO_4, 1.9; $Na_2HPO_4{\cdot}2H_2O$, 6.4;

NH_4Cl, 0.3; $MgCl_2{\cdot}6\ H_2O$, 0.1; NaCl, 0.3; KCl, 0.15; $CaCl_2{\cdot}2H_2O$, 0.055; and $Na_2S{\cdot}9H_2O$, 0.24; trace element solution, 2 mL (Chin et al., 1998); alkaline trace element solution 1 mL (Stams et al., 1993); vitamin solution, 1 mL (Wolin et al., 1963); resazurine at 0.5% (wt/vol), 1 mL; pH adjusted to 7.2. Glucose (Merck, Frankfurt, Germany; produced from maize starch), cellobiose (Fluka, Taufkirchen, Germany), or cellulose (Fluka, Taufkirchen, Germany; concentrations expressed as anhydroglucose; Mw = 162 g/mol) was used as substrate.

Isotope fractionation after complete conversion of saccharides was investigated in cultures of *C. papyrosolvens* (pure culture) and *C. papyrosolvens* with methanogenic partner (co-culture). Pure and co-culture experiments with glucose or cellobiose, in which the influence of temperature and substrate concentration was tested, were conducted in 120-mL serum vials with 50 mL of culture volume. Each combination of temperature and concentration was carried out in triplicate. Pure culture experiments with cellulose were performed only at 30 °C in 1000 mL glass bottles with 500 mL culture volume. The duration of batch experiments was between less than a week with glucose as substrate at 30 °C and up to 3 months with cellulose. Complete substrate conversion was confirmed by HPLC (see below) for glucose and cellobiose and by product accumulation for cellulose. After complete consumption, gas and liquid samples were taken for analysis of concentration and carbon isotopic composition. In addition, the co-culture of *C. papyrosolvens* and *M. bryantii* was grown on cellulose in triplicate (500 mL glass bottles; 250 mL culture volume) and continuously sampled during the course of growth.

Isotope fractionation during the linear part of fermentation (saccharide to pyruvate) was investigated at 30 °C in 1000 mL glass bottles with 500 mL culture volume in three replicate pure cultures with glucose (2.78 mM), cellobiose (1.46 mM), and cellulose (2.81 mM), respectively. Samples were taken continuously until complete fermentation of the saccharides.

To investigate isotope fractionation at steady-state, *C. papyrosolvens* was grown in continuous culture at different growth rates (μ). The chemostat principle allowed to vary μ by changing the dilution rate (D), which is the volume of medium added per time and culture volume. At steady-state conditions D equals μ. Samples were only taken after establishment of steady-state conditions, and therefore μ instead of D is used throughout the text. Chemostat cultures were performed in 600-mL fermenter vessels with a working volume of 290 ml (headspace flushed with N_2; low hydrogen setup) or 270 ml (non-flushed headspace; high hydrogen setup). The mineral medium described above was used, but with half the concentration of phosphate. *C. papyrosolvens* was grown at glucose limitation (10 mM), and steady-state conditions were maintained for at least 10 volume changes, before gas and liquid samples were taken. Cultures were kept at a temperature of 30 °C and stirred with 200 rpm. The pH was kept constant at pH 7.2 by automatic titration with NaOH solution. The biomass density was measured as optical density at 600 nm (OD_{600}) using a photometer.

Liquid samples for HPLC and isotopic analysis were filtered through 0.2-μm membrane filters (REZIST 13/0.2 PTFE, Schleicher and Schuell, Dassel, Germany) and stored at −20 °C until analysis. For $\delta^{13}C$ analysis of biomass, cells were harvested by centrifugation (26,000*g*, 15 min, 4 °C), washed two times with phosphate buffer (50 mM, pH 7.2), and dried to constant weight at 105 °C.

2.2. Chemical and isotopic analysis

CH_4 and CO_2 were analyzed by gas chromatography using a flame ionization detector (Shimadzu, Kyoto, Japan). CO_2 was detected after conversion to CH_4 with a methanizer (Ni-catalyst at 350 °C, Chrompack, Middelburg, Netherlands). H_2 was analyzed by gas chromatography using a thermal conductivity detector (Shimadzu, Kyoto, Japan). Acetate, ethanol, formate, lactate, glucose, and cellobiose were measured by HPLC (Sykam, Gilching, Germany) with a refraction index and UV-detector, having a detection limit of 3–5 μM (Krumböck and Conrad, 1991). Glucose concentration in the chemostat experiments was measured photometrically (Bergmeyer et al., 1974).

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system that was purchased from Thermoquest (Bremen, Germany). The principle operation was described (Brand, 1996). The CH_4 and CO_2 in the gas samples (40–400 μL) were first separated in a Hewlett Packard 6890 gas chromatograph using a Pora Plot Q column (27.5 m length, 0.32 mm i.d.; 10 μm film thickness; Chrompack, Frankfurt, Germany) at 30 °C and He (99.996% purity; 2.6 mL/min) as carrier gas. After conversion of CH_4 to CO_2 in the Finnigan Standard GC Combustion Interface III the $^{13}C/^{12}C$ was analyzed in the IRMS (Finnigan MAT delta plus).

To measure the $\delta^{13}C$ of acetate (mean of both C atoms), 0.5–2 mL liquid sample was freeze-dried (α1–4, Christ, Osterode, Germany) and thereafter redissolved in 20–50 μL formic acid (0.1 M) in propanol. Tests showed that freeze-drying did not cause isotope fractionation of acetate at pH > 8. Acetate samples (1 μL) were analyzed with a Hewlett Packard 6890 gas chromatograph using a FFAP column (30 m length, 0.32 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, USA). The oven temperature was kept at 80 °C for 4 min and then increased by 20 °C/min to 240 °C. Reference gas was CO_2 (99.998% purity; Messer-Griessheim, Düsseldorf, Germany) calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max-Planck-Institut für Biogeochemie, Jena, Germany (courtesy of Dr. W.A. Brand) against NBS22 and USGS 24, and reported in the delta notation versus V-PDB: $\delta^{13}C = 10^3(R_{sa}/R_{st} - 1)$ with $R = {}^{13}C/{}^{12}C$ of sample (sa) and standard (st), respectively. The precision of repeated analysis of 1.3 nmol CH_4 and 1.36 nmol acetate was ±0.2‰ and ±0.1‰, respectively.

Isotopic measurement of glucose, formate, lactate, ethanol, and acetate was performed on a HPLC system (Spectra System P1000, Thermo Finnigan, San Jose, CA, USA; Mistral, Spark, Emmen, The Netherlands) equipped with an ion-exclusion column (Aminex HPX-87-H, Biorad) and coupled to Finnigan LC IsoLink (Thermo Electron Corporation, Bremen, Germany) as described by Krummen et al. (2004). Isotope ratios were detected on a Finnigan MAT delta plus advantage IRMS. Reference gas was CO_2 calibrated as described above. The precision of repeated analysis was ±0.2‰ when 30 nmol acetate was injected into the HPLC system.

The analysis of $\delta^{13}C$ of biomass and the saccharides was carried out at the Max-Planck-Institute for Biogeochemistry, Jena, Germany, with a EA-IRMS system consisting of an autosampler (AS 128, CE Instruments, Rodano, Italy), an elemental analyser (NA 1110 CN, CE Instruments) and an isotope ratio mass spectrometer (IRMS Delta^{plus} XL, Finnigan MAT, Bremen, Germany), coupled via a home-built interface (''ConFlo III''; Werner et al. (1999)) modified and supplemented as described in Brooks et al. (2003). The samples and the laboratory reference compounds (acetanilide and caffeine) were applied in solid samples tin capsules (Lüdi, purchased from IVA, Meerbusch, Germany). The standardisation scheme of the EA-IRMS measurements as well as the measurement strategy and the calculations for assigning the final $\delta^{13}C$-values on the V-PDB scale was analogous to that described by Werner and Brand (2001) on an elemental-analyzer-IRMS.

2.3. Calculations

Fractionation factors for a reaction A → B are defined after Hayes (1993):

$$\alpha_{A/B} = (\delta_A + 1000)/(\delta_B + 1000), \tag{1}$$

sometimes expressed as $\varepsilon \equiv 10^3(1-\alpha)$. Because total oxidized carbon was distributed among different carbon species (gaseous CO_2, dissolved CO_2, HCO_3^-, and CO_3^{2-}), $\delta^{13}C$ of total inorganic carbon (δ_{TIC}) could not be determined directly. This value was calculated by the following mass-balance equation:

$$\delta_{TIC} = X_g\delta_g + X_d\delta_d + X_b\delta_b + X_c\delta_c, \tag{2}$$

where X = mole fraction and δ = isotopic composition of the C of g = gaseous CO_2, d = dissolved CO_2, b = HCO_3^-, and c = CO_3^{2-}. The distribution of carbon among these species was calculated using solubility and equilibrium constants (Stumm and Morgan, 1995). δ_g was measured directly, the remaining isotopic compositions were calculated from the relevant equilibrium isotopic fractionation factors at 15 and 30 °C (Deines et al., 1974; Mook et al., 1974):

$$\delta_d = \alpha_{d/g}\delta_g + (\alpha_{d/g} - 1)1000, \tag{3}$$

$$\delta_b = \alpha_{b/g}\delta_g + (\alpha_{b/g} - 1)1000, \tag{4}$$

$$\delta_c = \alpha_{c/g}\delta_g + (\alpha_{c/g} - 1)1000. \tag{5}$$

Fractionation at branch points in the fermentation path was calculated based on the conservation of mass and the difference in fractionation observed between the two products, which is a valid approach for systems at equilibrium and under kinetic control. The following approximations—that are appropriate for carbon isotope fractionation—were used (Hayes, 2001):

$$\delta_p = \delta_r + (1 - f_p)\Omega_d, \tag{6}$$

$$\delta_q = \delta_r - f_p\Omega_d, \tag{7}$$

where r = reactant; p, q = products of the reaction at the branch point; f_p = fractional yield of p; $\Omega = [(\alpha_{r/p}/\alpha_{r/q}) - 1]10^3$, and d = designation of the branch point (e.g., pyruvate). In the corresponding diagram, where f_p is plotted against the isotopic composition of p and q, the straight lines represent the isotopic compositions of the C flow branching into p and q, and are vertically separated by Ω Eq. (8), the difference between the isotope effects.

$$\Omega = -\Delta\delta_q/f_p = \Delta\delta_p/(1 - f_p) = \delta_p - \delta_q, \tag{8}$$

where $\Delta\delta_q = (\delta_q - \delta_r)$ and $\Delta\delta_p = (\delta_p - \delta_r)$. Values of Ω can alternatively be determined by the slope of the regression line of each product. Disagreement between the differently calculated values of Ω would imply that the C-flow might not be as simple as assumed (e.g., more than two branches). The fractional yields are defined as the follows:

$$f_{ac} = \frac{n(\text{acetate})}{n(\text{acetate}) + n(\text{ethanol})}, \tag{9}$$

$$f_{lac} = \frac{n(\text{lactate})}{n(\text{lactate}) + n(\text{acetate}) + n(\text{ethanol})}, \tag{10}$$

where n = moles of the accumulated products.

An equation presented by Mariotti et al. (1981) was used to calculate the isotopic effect (ε) associated with the uptake and fermentation of saccharides to pyruvate

$$\delta_{pyr} = \delta_{sacc} - \varepsilon(1 - f)[\ln(1 - f)]/f, \tag{11}$$

where δ_{sacc} = isotope composition of the saccharide at the beginning; δ_{pyr} = isotope composition of pyruvate at the instance, when f was determined; and f = fractional yield of pyruvate based on consumption of the saccharide ($0 < f < 1$). Linear regression of δ_{pyr} against $(1 - f)[\ln(1 - f)]/f$ gives ε as the slope. Isotope signatures were available for acetate (δ_{ac}), but not for all the other fermentation products of pyruvate. Therefore, δ_{pyr} had to be calculated from the fractionation factors derived from the branch point analysis ($\Omega_{ac\text{-}CoA} = 17$; $\Omega_{pyr} = 4$). Average isotope composition of the products was calculated using Eqs. (12) and (13) based on mass balance equation:

$$\delta_{ac\text{-}CoA} = f_{ac}\delta_{ac} + (1 - f_{ac})(\delta_{ac} - \Omega_{ac\text{-}CoA}), \tag{12}$$

$$\delta_{pyr} = (1 - f_{lac})\delta_{ac\text{-}CoA} + f_{lac}(\delta_{ac\text{-}CoA} - \Omega_{pyr}). \tag{13}$$

3. Results

3.1. Batch experiments

Clostridium papyrosolvens converted the saccharides (sacc) glucose (glu), cellobiose, and cellulose in a mixed-acid fermentation to the major products acetate (ac), ethanol (etoh), H_2 and CO_2 (Table 1). Additional minor fermentation products were formate (form) and lactate (lac). Carbon recoveries were $108.8 \pm 4.1\%$, $68.7 \pm 1.9\%$, and $64.3 \pm 0.5\%$ for glucose, cellobiose, and cellulose, respectively. Since the saccharides were completely consumed, the residual carbon must have been converted into microbial biomass (biom). The biomass yield was apparently higher with cellobiose and cellulose, which is consistent with carbon recoveries in *Clostridium thermocellum* (Strobel, 1995) and *Ruminococcus albus* (Thurston et al., 1993), where cell yields were 30% higher for cellobiose than for glucose as substrate. In *Clostridium cellulolyticum*, which is closely related to *C. papyrosolvens* (96.6% 16S rDNA gene similarity), cell yields were found to be higher with cellulose than with cellobiose (Desvaux et al., 2000). The increased biomass yield is explained by the expense of less ATP per hexose unit when cellulose or cellobiose rather than glucose are the substrate (Lynd et al., 2002).

In Table 1, the patterns of product formation for the different substrates and different growth conditions illustrate the metabolic flexibility of *C. papyrosolvens*. Fermentation of glucose resulted in lower amounts of H_2 at 30 °C versus 15 °C at all substrate concentrations, and resulted instead in the accumulation of increased amounts of formate. While ethanol was an important product on glucose and cellobiose, lactate was only produced in higher amounts on cellobiose. Different saccharide concentrations did not influence the pattern of product formation on glucose, but on cellobiose f_{lac} increased with the saccharide concentration (Table 2). The fermentation pattern on cellulose was similar as on glucose, but no formate was produced.

In addition to the pure culture experiments, *C. papyrosolvens* was also grown in methanogenic co-culture with either *M. hungatei* or *M. bryantii* (Table 1). *M. hungatei* and *M. bryantii* are both hydrogenotrophic methanogens, i.e., they produce CH_4 from H_2/CO_2. *M. hungatei* also uses formate as alternative substrate for CH_4 production. However, they do not use acetate for CH_4 production and assimilate only small amounts of it into biomass. To be able to generate a carbon mass balance for *C. papyrosolvens*, total inorganic carbon (TIC) produced during fermentation was calculated as the sum of produced methane and remaining TIC at the end of the incubation. Methane produced by methanogenic archaea increased with saccharide concentration in both co-cultures. Methane yields were lower in the co-cultures with *M. bryantii* compared to those with *M. hungatei*, because formate was not metabolized by *M. bryantii* and could accumulate in the system. Carbon recoveries were $90.4 \pm 3.4\%$ and $69.3 \pm 2.8\%$ for glucose and cellobiose, respectively, showing the same trend as in pure culture (Table 1).

Interestingly, the product patterns of the co-cultures were strongly different between the different saccharides. With glucose, the carbon flow to lactate increased strongly with substrate concentration and reached up to 20% of initial glucose carbon at the highest concentration. The f_{ac} and f_{lac} in the co-culture experiments were larger than those in the pure culture experiments indicating that the electron flow was preferentially into lactate rather than ethanol (Table 2). With cellobiose, the fermentation pattern in co-culture was similar as in pure culture. Slightly lower yields of formate in the co-culture with *M. bryantii* could be due to the consumption of produced H_2 and the concomitantly lower conversion of $H_2 + CO_2$ to formate. Co-cultures on cellulose were only performed with *M. bryantii* and were not run to completion, as it shown by still ongoing product formation (Fig. 2). In the beginning (<600 h), H_2 accumulated and the fermentation pattern was similar as observed in pure culture. Later on, the H_2 partial pressure (p_{H2}) decreased and f_{ac} increased from 0.68 to values larger than 0.8 (Fig. 2). This effect of p_{H2} on f_{ac} was also observed in pure culture (relation included in Fig. 3).

In pure cultures of *C. papyrosolvens*, acetate was generally enriched in ^{13}C ($\alpha_{sacc/ac} = 0.991$–0.997), with the smallest fractionation on cellulose (Table 2). Temperature did not influence the fractionation factor, whereas increasing cellobiose concentrations resulted in decreasing fractionation ($\alpha_{sacc/ac}$ increased from 0.991 to 0.995). The TIC formed from glucose was slightly enriched in ^{13}C or was not fractionated, while that from cellobiose exhibited a stronger enrichment in ^{13}C (Table 2). For biomass formation ^{12}C was generally preferred with a slightly stronger fractionation for glucose than for cellobiose. In the methanogenic co-cultures, only the fractionation of saccharide into acetate could be evaluated, since TIC was enriched in ^{13}C due to the strong isotope effect associated with hydrogenotrophic methanogenesis (Table 2). Independent of the methanogenic partner and the saccharide concentration, $\alpha_{sacc/ac} = 1.000$–1.002 and $\alpha_{sacc/ac} = 0.995$–0.996 was observed for glucose and cellobiose, respectively. In the methanogenic co-cultures grown on cellulose, δ_{ac} was initially similar to that observed in pure culture, but eventually decreased with increasing f_{ac}, becoming close to that of $\delta_{cellulose}$ (Fig. 2).

In separate batch pure culture experiments the isotope fractionation during incomplete fermentation was investigated. While the products and concentrations were as described for the batch pure cultures above, δ_{ac} showed a different trend with cellulose than with the soluble saccharides glucose and cellobiose (data not explicitly shown). With cellulose δ_{ac} stayed fairly constant, whereas with glucose and cellobiose it increased with time until the saccharides were completely consumed. These data are used in Section 3.3 for analysis of fractionation from saccharide to pyruvate.

Table 1
Product formation and carbon recovery during fermentation of different saccharides by *C. papyrosolvens* in batch pure cultures and co-cultures with hydrogenotrophic methanogenic archaea

Substrate	Organisms	T (°C)	Saccharide (mM)	Lactate (mmol)	Formate (mmol)	Acetate (mmol)	Ethanol (mmol)	TIC (mmol)	Hydrogen (mmol)	Methane (mmol)	Carbon recovery (%)
Glucose ($\delta^{13}C = -10.67‰$)	*C. papyrosolvens*	15	2.22	0.002 ± 0.000	0.017 ± 0.001	0.187 ± 0.006	0.064 ± 0.003	0.282 ± 0.010	0.309 ± 0.004		121.1
			4.44	0.001 ± 0.001	0.015 ± 0.003	0.357 ± 0.018	0.098 ± 0.004	0.575 ± 0.040	0.651 ± 0.035		112.8
			8.88	0.001 ± 0.001	0.010 ± 0.000	0.602 ± 0.001	0.158 ± 0.014	1.044 ± 0.027	1.132 ± 0.002		98.0
	C. papyrosolvens	30	2.22	0.000 ± 0.000	0.039 ± 0.001	0.143 ± 0.004	0.092 ± 0.001	0.284 ± 0.006	0.217 ± 0.002		119.0
			4.44	0.000 ± 0.000	0.084 ± 0.001	0.238 ± 0.002	0.166 ± 0.002	0.447 ± 0.017	0.368 ± 0.003		100.7
			8.88	0.010 ± 0.001	0.168 ± 0.001	0.407 ± 0.005	0.336 ± 0.006	0.907 ± 0.032	0.635 ± 0.010		101.1
Cellobiose ($\delta^{13}C = -25.11‰$)	*C. papyrosolvens*	30	1.17	0.006 ± 0.000	0.020 ± 0.002	0.140 ± 0.001	0.070 ± 0.001	0.143 ± 0.006	0.160 ± 0.001		68.7
			2.34	0.022 ± 0.001	0.073 ± 0.002	0.357 ± 0.001	0.089 ± 0.002	0.254 ± 0.005	0.287 ± 0.003		73.2
			4.67	0.090 ± 0.004	0.151 ± 0.005	0.522 ± 0.013	0.155 ± 0.017	0.432 ± 0.017	0.482 ± 0.004		64.0
Cellulose ($\delta^{13}C = -25.23‰$)	*C. papyrosolvens*	30	2.81[a]	0.014 ± 0.003	ND	1.344 ± 0.005	0.587 ± 0.023	1.540 ± 0.010	3.787 ± 0.050		64.3
Glucose ($\delta^{13}C = -10.67‰$)	*C. papyrosolvens*/*M. hungatei*	30	2.22	0.031 ± 0.002	ND	0.119 ± 0.005	0.037 ± 0.001	0.271 ± 0.006	0.00 ± 0.000	0.07 ± 0.003	84.9
			4.44	0.074 ± 0.024	ND	0.221 ± 0.003	0.069 ± 0.008	0.370 ± 0.008	0.00 ± 0.000	0.13 ± 0.003	80.9
			8.88	0.191 ± 0.017	ND	0.470 ± 0.021	0.133 ± 0.017	0.600 ± 0.056	0.00 ± 0.001	0.26 ± 0.014	89.3
	C. papyrosolvens/*M. bryantii*	30	2.22	0.026 ± 0.011	0.055 ± 0.006	0.130 ± 0.003	0.038 ± 0.004	0.492 ± 0.019	0.00 ± 0.000	0.06 ± 0.004	105.3
			4.44	0.089 ± 0.024	0.072 ± 0.031	0.230 ± 0.012	0.044 ± 0.015	0.582 ± 0.052	0.00 ± 0.000	0.12 ± 0.017	92.7
			8.88	0.211 ± 0.015	0.162 ± 0.007	0.407 ± 0.001	0.099 ± 0.008	0.631 ± 0.017	0.00 ± 0.000	0.22 ± 0.004	89.3
Cellobiose ($\delta^{13}C = -25.11‰$)	*C. papyrosolvens*/*M. hungatei*	30	1.17	0.024 ± 0.009	ND	0.113 ± 0.004	0.035 ± 0.001	0.264 ± 0.006	0.00 ± 0.000	0.06 ± 0.002	73.3
			2.34	0.013 ± 0.007	ND	0.193 ± 0.002	0.092 ± 0.004	0.356 ± 0.006	0.00 ± 0.000	0.13 ± 0.002	62.5
			4.67	0.033 ± 0.005	ND	0.381 ± 0.020	0.203 ± 0.012	0.600 ± 0.005	0.00 ± 0.000	0.24 ± 0.007	62.6
	C. papyrosolvens/*M. bryantii*	30	1.17	0.007 ± 0.004	0.019 ± 0.005	0.116 ± 0.006	0.036 ± 0.004	0.506 ± 0.014	0.00 ± 0.000	0.07 ± 0.003	80.7
			2.34	0.012 ± 0.008	0.066 ± 0.013	0.192 ± 0.005	0.086 ± 0.002	0.567 ± 0.005	0.00 ± 0.000	0.11 ± 0.003	69.8
			4.67	0.041 ± 0.005	0.164 ± 0.023	0.341 ± 0.009	0.183 ± 0.015	0.686 ± 0.006	0.00 ± 0.000	0.19 ± 0.006	66.9

ND, not detected.
[a] In hexose equivalents.

Table 2
Isotope ratios and fractionation factors of products of fermentation of different saccharides by *C. papyrosolvens* in batch pure cultures and co-cultures with hydrogenotrophic methanogenic archaea

Substrate	Organisms	T (°C)	Saccharide (mM)	f_{ac}[b]	f_{lac}[b]	pH	δ_{ac} (‰)	δ_{etoh}	δ_{lac}	δ_{TIC} (‰)	δ_{biom} (‰)	$\delta_{acetyl\text{-}CoA}$ (‰)[a]	$\alpha_{sacc/ac}$	$\alpha_{sacc/TIC}$	$\alpha_{sacc/biom}$
Glucose ($\delta^{13}C = -10.67‰$)	*C. papyrosolvents*	15	2.22	0.74	0.01	7.02 ± 0.01	-6.72 ± 1.87	-22.84 ± 0.22	-13.71 ± 1.64	-9.78 ± 1.02	-14.06 ± 0.16	−10.38	0.996	0.999	1.000
			4.44	0.79	0.00	6.86 ± 0.01	-4.98 ± 0.27	-23.68 ± 0.19	-15.30 ± 0.34	-9.54 ± 0.23	-16.09 ± 0.13	−9.31	0.994	0.999	0.999
			8.88	0.79	0.00	6.66 ± 0.01	-4.27 ± 0.11	-23.31 ± 0.36	-16.77 ± 0.24	-10.91 ± 0.35	-16.52 ± 0.16	−8.62	0.994	1.000	0.998
	C. papyrosolvents	30	2.22	0.61	0.00	7.02 ± 0.01	-4.17 ± 0.43	-19.41 ± 0.14	-13.28 ± 1.12	-8.43 ± 0.14	-14.67 ± 1.35	−10.65	0.993	0.998	1.000
			4.44	0.59	0.00	6.86 ± 0.01	-2.69 ± 0.30	-18.33 ± 0.50	-12.40 ± 0.05	-7.30 ± 0.31	-12.10 ± 0.14	−10.04	0.992	0.997	0.999
			8.88	0.55	0.01	6.66 ± 0.01	-4.21 ± 0.22	-17.59 ± 0.06	-12.28 ± 0.07	-7.30 ± 0.14	-12.09 ± 0.04	−10.54	0.994	0.997	1.000
Cellobiose ($\delta^{13}C = -25.11‰$)	*C. papyrosolvents*	30	1.17	0.66	0.03	7.04 ± 0.01	-16.32 ± 0.86	NA	NA	-19.70 ± 0.31	-25.17 ± 0.15		0.991	0.994	0.975
			2.34	0.80	0.05	6.93 ± 0.01	-18.45 ± 0.55	NA	NA	-18.89 ± 0.11	-26.24 ± 0.00		0.993	0.994	0.975
			4.67	0.77	0.12	6.64 ± 0.02	-20.58 ± 0.52	NA	NA	-18.34 ± 0.09	-26.80 ± 0.11		0.995	0.993	0.975
Cellulose ($\delta^{13}C = -25.23‰$)	*C. papyrosolvents*	30	2.81	0.70	0.01	6.950 ± 0.02	-22.46 ± 0.25	NA	NA	-22.61 ± 0.03			0.997	0.997	
Glucose ($\delta^{13}C = -10.67‰$)	*C. papyrosolvents*/ *M. hungatei*	30	2.22	0.76	0.17	7.07 ± 0.00	-12.23 ± 1.39	NA	NA	-3.63 ± 0.07			1.002		
			4.44	0.76	0.20	6.92 ± 0.02	-10.70 ± 0.35	NA	NA	-1.23 ± 0.22			1.000		
			8.88	0.78	0.24	6.62 ± 0.01	-10.67 ± 0.41	NA	NA	1.10 ± 0.83			1.000		
	C. papyrosolvents/ *M. bryantii*	30	2.22	0.77	0.13	7.07 ± 0.03	-10.78 ± 0.54	NA	NA	-4.77 ± 0.66			1.000		
			4.44	0.84	0.25	6.93 ± 0.01	-11.97 ± 0.88	NA	NA	-2.53 ± 0.65			1.001		
			8.88	0.80	0.29	6.72 ± 0.01	-12.05 ± 0.04	NA	NA	4.36 ± 0.30			1.001		
Cellobiose ($\delta^{13}C = -25.11‰$)	*C. papyrosolvens*/ *M. hungatei*	30	1.17	0.76	0.14	7.04 ± 0.01	-20.22 ± 0.27	NA	NA	-10.59 ± 0.62			0.995		
			2.34	0.68	0.05	6.92 ± 0.01	-21.59 ± 0.12	NA	NA	-10.98 ± 0.27			0.996		
			4.67	0.65	0.05	6.64 ± 0.01	-21.10 ± 0.27	NA	NA	-9.68 ± 0.70			0.996		
	C. papyrosolvents/ *M. bryantii*	30	1.17	0.76	0.05	7.13 ± 0.01	-21.40 ± 0.74	NA	NA	-10.61 ± 0.18			0.996		
			2.34	0.69	0.04	6.99 ± 0.01	-21.10 ± 0.56	NA	NA	-12.55 ± 1.60			0.996		
			4.67	0.65	0.07	6.72 ± 0.02	-20.09 ± 0.50	NA	NA	-9.60 ± 1.23			0.995		

NA, not analyzed.
[a] Calculated using Eq. (17).
[b] f_{ac} and f_{lac} were calculated from Table 1 using Eqs. (9) and (10), respectively.

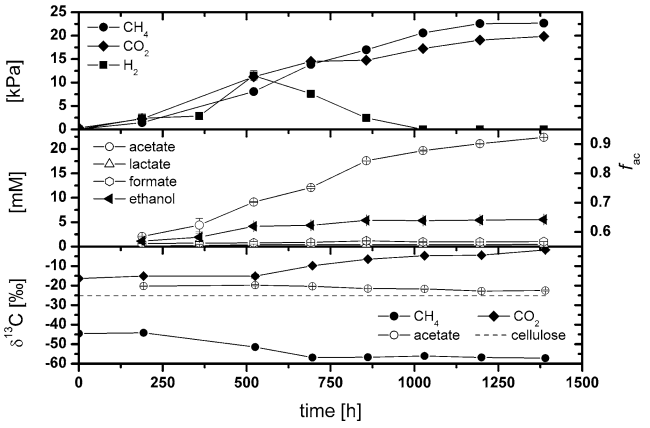

Fig. 2. Co-culture of *C. papyrosolvens* and *M. bryantii* growing on cellulose as the only substrate. After a lag phase hydrogen formed by *C. papyrosolvens* was consumed by *M. bryantii* leading to a shift of the fermentation pattern to acetate (increasing f_{ac}) concomitant with a decrease in δ_{ac}. Strong fractionation of methanogenesis causes ^{13}C enrichment of CO_2. All measured compounds are given as mean ± SE, $n = 3$.

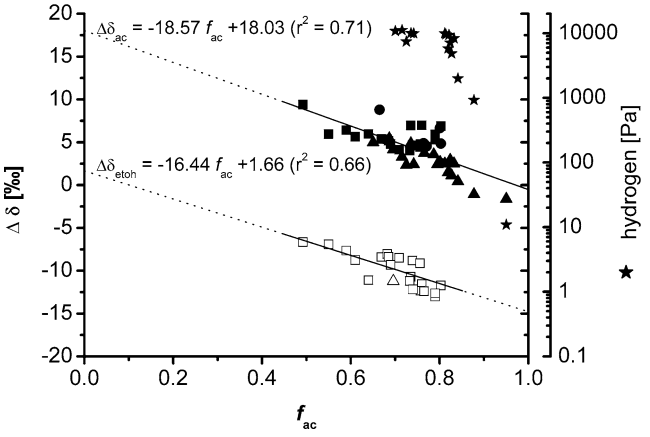

Fig. 3. Split of isotope carbon flow at the branch point acetyl-CoA (see scheme in Fig. 1). Fractionation is shown by the slope and the vertical difference between the product regression lines (regression lines drawn beyond data points are dotted). Strong isotope fractionation occurred between acetate and ethanol, where acetate was the ^{13}C-enriched product. f_{ac} increased with decreasing p_{H2} for *C. papyrosolvens* grown on cellulose. Δδ-values of acetate and ethanol are shown for batch and chemostat experiments with $f_{lac} < 0.05$ (see Tables 2 and 3): ■, acetate from glucose; □, ethanol from glucose; ●, acetate from cellobiose; ▲, acetate from cellulose; △, ethanol from cellulose; ★, H_2 from cellulose.

3.2. Chemostat experiments

Clostridium papyrosolvens was grown in glucose-limited chemostat culture under a relatively high (64.10 ± 5.12%) and a relatively low H_2 mixing ratio (0.59 ± 0.18%). Concentration and isotopic composition of the fermentation products, turbidity of the culture (OD_{600}), and fractionation factors are shown for $0.02 \leqslant \mu \leqslant 0.25\ h^{-1}$ in Fig. 4 and Table 3. To be able to generate a carbon mass balance (Table 3), TIC produced during fermentation was calculated by

$$n(\mathrm{TIC}) = \frac{n(\mathrm{glucose}) - n(\mathrm{lactate})}{3} - n(\mathrm{formate}), \qquad (14)$$

where n is given as mol of carbon.

At $\mu \leqslant 0.15\ h^{-1}$, glucose was completely fermented, whereas at higher μ, the bacteria were not able to completely consume glucose (neither under high nor low p_{H2}), which finally resulted in washout of the cells at $\mu = 0.25\ h^{-1}$. The product pattern was similar as in the batch cultures with glucose and did not significantly change with μ. Absolute amounts of the products, however, varied with the chemostat setup. Slightly higher amounts of ethanol, lactate, and formate were observed at the higher p_{H2} (Fig. 4A), indicating an increased electron flow into reduced fermentation products rather than into acetate. The fractionation to biomass was independent of growth rate and extent of glucose consumption, with $\alpha_{sacc/biom} = 1.001–1.002$.

At $\mu \leqslant 0.15\ h^{-1}$ (complete consumption of glucose), acetate was generally ^{13}C-enriched giving a range of $\alpha_{glu/ac} = 0.991–0.996$ (Table 3). The reduced products lactate and ethanol were ^{12}C-enriched ($\alpha_{glu/lac} = 1.001–1.006$; $\alpha_{sacc/etoh} = 1.007–1.013$). At low p_{H2}, fractionation of glucose to carbon dioxide (here δ_{CO_2} rather than δ_{TIC} was used, since equilibrium between the inorganic carbon species did not establish) decreased the $^{13}C/^{12}C$ ratio ($\alpha_{glu/CO_2} = 1.000–1.005$). At high p_{H2}, δ_{CO_2} steadily increased with time, and did not stabilize within ten volume changes. Therefore we tested this increase over an extended period at a growth rate of $\mu = 0.075$. Equilibrium was finally reached after 180 volume changes. The delayed adjustment of the equilibrium was probably due to equilibration between CO_2 and formate and was properly described by a logarithmic function of δ_{CO_2} with volume changes (Fig. 5). Fractionation between CO_2 and formate was calculated from the last three data points in Fig. 5 ($\alpha_{CO_2/form} = 1.015$). At dilution rates > $0.15\ h^{-1}$ (incomplete consumption of glucose) and at high p_{H2},fractionation of ^{13}C was similar as in the other experiments.

3.3. Data analysis

The analysis of isotopic behavior at the branch points (Fig. 1) of the fermentation pathway was based on the Eqs. (6) and (7) (Hayes, 2001). The data are plotted for the branch point acetyl-CoA (ac-CoA) (Fig. 3). The fractional yield (f) of one of the formed products is plotted against the difference of the isotopic values of product and substrate ($\Delta\delta$). A negative slope indicates that f represents the fraction of the product enriched in ^{13}C and vice versa. For the branch point acetyl-CoA, $\delta_r = \delta_{ac\text{-}CoA}$, the ^{13}C-enriched product was acetate and the ^{13}C-depleted product was ethanol. In these experiments, in which only a small fraction of the carbon flow branched into lactate at the upstream branch point pyruvate (Fig. 1), it can be assumed that $\delta_{ac\text{-}CoA} = \delta_{sacc}$. Therefore, we considered for the data analysis only experiments, in which $f_{lac} < 0.05$ (Fig. 3). We also assumed that the branching of carbon to anabolic reactions had a

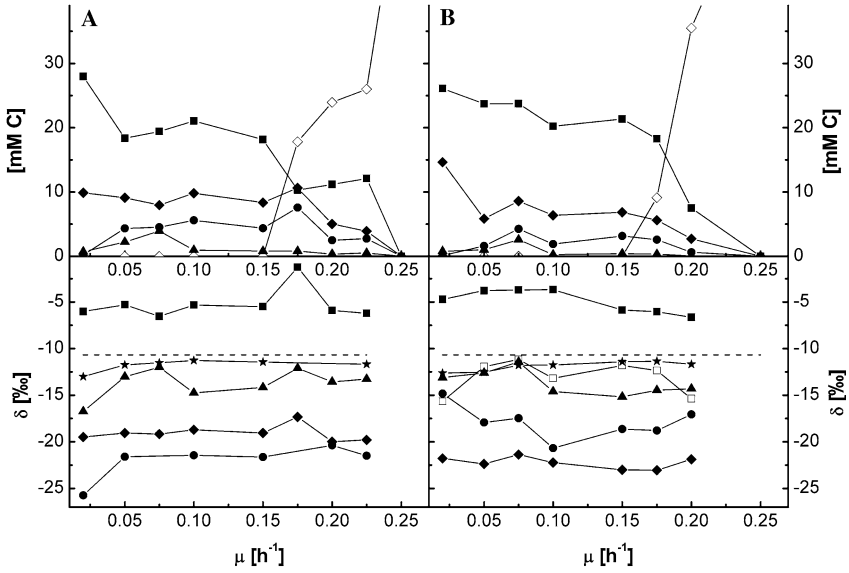

Fig. 4. Glucose-limited chemostat culture of *C. papyrosolvens* at $0.02 \leqslant \mu \leqslant 0.25\ h^{-1}$. Upper panels show steady-state concentrations of glucose and dissolved products in the chemostat, lower panels show the carbon isotope composition of biomass and products. (A) high hydrogen setup, (B) low hydrogen setup. ◇, glucose; ■, acetate; ♦, ethanol; ▲, lactate; ●, formate; □, carbon dioxide; ★, biomass; dashed line, initial δ_{glu}.

Table 3
Growth and isotope fractionation recorded in glucose-limited chemostat cultures of *C. papyrosolvens*

Hydrogen concentration	μ (h^{-1})	OD_{600}	Carbon recovery (%)	δ_{lac} (‰)	$\delta_{acetyl\text{-}CoA}$ (‰)[a]	$\alpha_{glu/ac}$	$\alpha_{glu/lactate}$	$\alpha_{glu/ethanol}$	α_{glu/CO_2}	$\alpha_{glu/biomass}$
High	0.020	0.333	97.6	−16.74	−9.53	0.995	1.006	1.009	1.005	1.002
	0.050	0.481	82.8	−13.01	−9.85	0.995	1.002	1.009	1.000	1.001
	0.075	0.701	85.5	−12.00	−10.21	0.996	1.001	1.009	0.998	1.001
	0.100	0.840	86.4	−14.74	−9.59	0.995	1.004	1.008	0.997	1.001
	0.150	0.660	78.8	−14.16	−9.76	0.995	1.004	1.009	0.998	1.001
	0.175	0.536	89.3	−12.11	−9.44	0.991	1.001	1.007		
	0.200	0.556	87.5	−13.56	−10.26	0.995	1.003	1.010	0.997	1.001
	0.225	0.544	89.7	−13.27	−9.52	0.996	1.003	1.009	0.998	
Low	0.020	0.367	102.5	−13.11	−10.84	0.994	1.002	1.011	1.005	1.002
	0.050	0.400	84.2	−12.60	−7.45	0.993	1.002	1.012	1.001	1.002
	0.075	0.692	91.5	−11.43	−8.40	0.993	1.001	1.011	1.000	1.001
	0.100	0.644	78.1	−14.60	−8.13	0.993	1.004	1.012	1.003	1.001
	0.150	0.810	81.0	−15.17	−10.01	0.995	1.005	1.013	1.001	1.001
	0.175	0.800	83.8	−14.46	−10.04	0.995	1.004	1.013	1.002	1.001
	0.200	0.316	89.9	−14.31	−10.70	0.996	1.004	1.011	1.005	1.001

[a] Calculated using Eq. (17).

negligible effect on carbon isotope fractionation since $\alpha_{sacc/biom} \approx 1$ (Tables 2 and 3). The value of $\Omega_{ac\text{-}CoA}$ at the branch point acetyl-CoA is given by the difference between δ_{ac} and δ_{etoh} Eq. (8) resulting in $\Omega_{ac\text{-}CoA} = 15.45 \pm 2.01$ (average ± SD). Alternatively, $\Omega_{ac\text{-}CoA}$ was computed from the slope of the regression lines in Fig. 3 Eq. (7), giving:

$$\Delta\delta_{ac} = (\delta_{ac\text{-}CoA} - \delta_{ac}) = -18.57 f_{ac} + 18.03 \quad (r^2 = 0.71; \mathrm{SD} = 1.58) \tag{15}$$

$$\Delta\delta_{etoh} = (\delta_{ac\text{-}CoA} - \delta_{etoh}) = -16.44 f_{ac} + 1.66 \quad kip1em(r^2 = 0.66; \mathrm{SD} = 1.35). \tag{16}$$

$\Omega_{ac\text{-}CoA}$ determined from the slopes of Eqs. (15) and (16) agrees well, and was only slightly higher than the $\Omega_{ac\text{-}CoA}$ determined by difference.

For saccharide fermentation via the EMP pathway the carbon atoms in acetyl-CoA correspond to positions 1,2,5, and 6 in the glucose-units. Thus, the difference in $\delta_{C1,2,5,6}$ and δ_{sacc} can theoretically be seen as the intercept of the regression line at $f_{ac} = 1$. However, the accuracy of the data was too low for interpretation of the intramolecular composition of the saccharides and its effect on isotope fractionation.

For the branch point pyruvate (Fig. 1), lactate and acetyl-CoA + CO_2 were the alternative products. Assuming a statistical isotope distribution in the saccharides, we can determine Ω analogous to the branch point acetyl-CoA using $\delta_r = \delta_{sacc}$, $\delta_p = \delta_{lac}$, and $\delta_q = \delta_{ac\text{-}CoA}$. $\delta_{ac\text{-}CoA}$ was calculated by the following mass balance equation:

$$\delta_{ac\text{-}CoA} = f_{ac}\delta_{ac} + (1 - f_{ac})\delta_{etoh}. \tag{17}$$

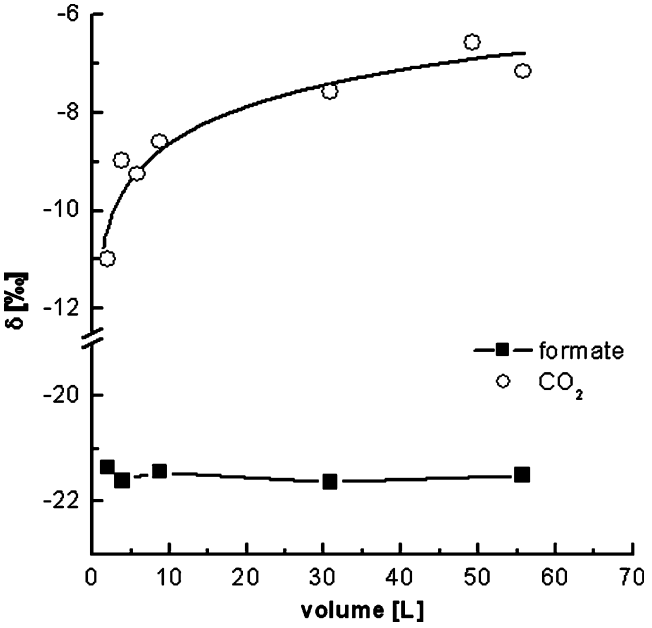

Fig. 5. Establishment of isotopic equilibrium between CO_2 and formate in the non-flushed headspace chemostat (high hydrogen setup) at $\mu = 0.075$. Non-steady-state conditions in the headspace versus steady-state conditions in the liquid phase allows ^{13}C enrichment of CO_2 until equilibrium, which is described by a logarithmic curve. The volume on the x-axis is the volume of medium added to the chemostat during the experiment.

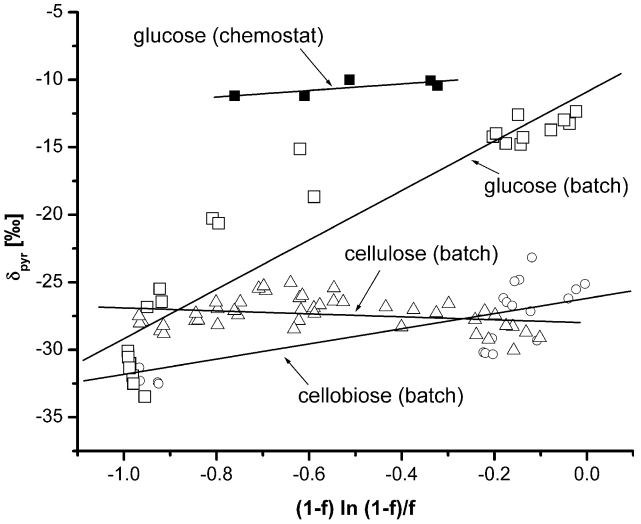

Fig. 6. Isotope fractionation by *C. papyrosolvens* during the course of fermentation of different saccharides to pyruvate in batch and chemostat culture fitted to the equation derived by Mariotti et al. (1981). δ_{pyr} is the isotope ratio of all products, calculated by Eqs. (12) and (13). Batch experiments: □, glucose; ○ cellobiose; △, cellulose. Chemostat experiment: ■, glucose at $\mu > 0.15$. Lines are linear regressions for the different experiments.

The assumption allows to determine the fractionation behavior at the branch point without using the isotope signature of the different inorganic carbon species and formate. This is very helpful, since a relatively strong kinetic isotope effect is associated with the formation of formate from CO_2, so that the isotope ratio of all the carbon species formed from CO_2 could not be determined. Generally lactate was depleted in ^{13}C in comparison to acetyl-CoA (Tables 2 and 3). Due to the low range of f_{lac}, which was observed in the experiments were δ_{lac} and δ_{etoh} were explicitly measured, the value of $\Omega_{pyruvate}$ at the branch point could not be determined by regression Eqs. (6) and (7) and was only determined from the difference between $\delta_{ac\text{-}CoA}$ and δ_{lac} Eq. (8), giving $\Omega_{pyr} = 4.08 \pm 1.79$ (average ± SD). Since Ω_{pyr} was relatively small, the experimental and analytical error had a stronger influence on Ω_{pyr} than in the case of $\Omega_{ac\text{-}CoA}$ at the downstream branch point. However, $\delta_{ac\text{-}CoA}$ for $f_{lac} \approx 0$ almost equaled the initial δ_{glu}, which is in agreement with the assumption of no change in isotopic composition from the saccharide to pyruvate. The preferential isotope flow through the fermentation network of *C. papyrosolvens* is schematically summarized in Fig. 1.

To address the question, whether there is a potential fractionation associated with the linear reactions involved in uptake of saccharide and catabolism to pyruvate, the first branch point, we evaluated those data, in which the saccharide substrate was not completely consumed, using Eq. (11) derived by Mariotti et al. (1981). For this purpose $\delta_{pyruvate}$ was calculated for batch and chemostat experiments using Eqs. (12) and (13), based on the mass balance equation and the fractionation factors (Ω) determined at the branch points.

In Fig. 6, δ_{pyr} is plotted as a function of $(1-f)[\ln(1-f)]/f$, with f = fraction of saccharide consumed. In batch experiments the ε determined from the slope was $18.29 \pm 2.96‰$ and 5.62 ± 1.91 (mean ± SD) for glucose and cellobiose, respectively. After quantitative conversion of the saccharide (i.e., $f = 1$; $(1-f)[\ln(1-f)]/f = 0$), the δ_{pyr} was $-10.89‰$ and $-26.19‰$ for glucose and cellobiose, respectively, as determined by extrapolation of the regression line. This is in good agreement with δ_{sacc} of the respective substrate ($\delta_{glu} = -10.67$; $\delta_{cellobiose} = -25.11$). For cellulose in batch, δ_{pyr} did not change with $(1-f)[\ln(1-f)]/f$ ($\varepsilon = -1.11 \pm 1.11$; mean ± SD), which means that no fractionation occurred during uptake and catabolism to pyruvate. In addition to the batch experiments, this analysis was also done for chemostat experiments, in which glucose was not completely consumed at steady-state conditions (Fig. 6). Values of δ_{pyr} were close to δ_{glu} and gave only indication of very low fractionation ($\varepsilon = 2.41 \pm 0.44$; mean ± SD).

4. Discussion

Stable carbon isotope fractionation during fermentation of saccharides was studied in *C. papyrosolvens*. Isotope fractionation can occur in linear reactions, such as substrate uptake, if the substrate is not completely consumed. More importantly, however, isotope fractionation generally occurs at branch points in the metabolic path.

4.1. Isotope fractionation during the linear part of the metabolic pathway

Saccharides are linearly metabolized in *C. papyrosolvens* to the first branch point of the fermentation path, i.e., pyruvate (Fig. 1). Fractionation between saccharide and pyruvate was found in batch experiments for the soluble saccharides glucose and cellobiose, but not for insoluble cellulose (Fig. 6). There, fractionation was stronger for glucose than for cellobiose, but δ_{pyr} derived from glucose deviated from the linear Mariotti equation (Fig. 6). Furthermore, results from glucose fermentation were different for batch and chemostat conditions (Fig. 6). At steady-state conditions in the chemostat at $\mu > 0.15\ h^{-1}$, conversion of glucose to pyruvate resulted in a very low fractionation. We do not know the reasons for the different fractionation patterns observed for the conversion of glucose to pyruvate under the different growth conditions. We speculate that transient formation of storage products could have influenced isotope fractionation. For example, *C. celluloyticum* was shown to form glycogen predominantly during the exponential growth phase (Guedon et al., 2000). Hence, it is possible that an additional carbon branch to glycogen in *C. papyrosolvens* caused the non-linear relation in the Mariotti plot. For cellulose utilization, however, no such fractionation was observed (Fig. 6). Cellulose is hydrolyzed at the cellulosome by expenditure of ATP (Lynd et al., 2002), and the resulting oligomers and glucose are then transported into the cell. We assume, that after cellulose hydrolysis the products are taken up before a larger pool of molecules establishes, so that uptake happens quantitatively without fractionation. A rather fast uptake appears to be a reasonable strategy for the microbial cell, as it avoids giving competitors access to the easily degradable hydrolysis products. In natural anaerobic systems glucose is usually present at only very low concentrations (King and Klug, 1982; Chidthaisong et al., 1999), so that quantitative uptake of the products of polysaccharide hydrolysis without isotope fractionation may be the rule. Indeed, hydrolysis of polysaccharides has been found to be the rate-limiting step in the degradation of complex organic matter (Degens and Mopper, 1975; Billen, 1982; Fey and Conrad, 2003). Therefore, it is reasonable to assume that fractionation during uptake of saccharides does not occur in natural environments. Consequently, experiments with quantitative conversion of saccharides are adequate for investigation of the stable carbon isotope fractionation associated with the branch points in the fermentation path.

4.2. Division of carbon isotopes at branches in the metabolic pathway

Since in *C. papyrosolvens* the fermentation path of saccharides is linear until the formation of pyruvate, δ_{pyr}—integrated over the complete fermentation—equals δ_{sacc}. At the first branch point pyruvate, the carbon flow is channeled to the products lactate, acetyl-CoA + CO_2, and biomass. Lactate was generally ^{13}C-depleted with respect to acetyl-CoA, indicating a stronger preference for ^{12}C by the lactate dehydrogenase than the pyruvate ferredoxin oxidoreductase (Tables 2 and 3). The saccharides were assumed to have a homogenous intramolecular isotopic composition. Yet for glucose produced from maize, which was used in this study, carbon positions (i.e., C-1, 2, 5, 6) forming acetyl-CoA were found to be ^{13}C-depleted ($\delta_{sacc} - \delta_{C1,2,5,6} = 1.09‰$) compared to total glucose (Rossmann et al., 1991). Therefore, Ω_{pyr} determined by the vertical difference between δ_{lac} and $\delta_{ac\text{-}CoA}$ would be affected. If we consider the depletion in ^{13}C (i.e., 2.18‰) at the positions in the glucose that are later converted to acetyl-CoA and the difference between the δ_{lac} and $\delta_{ac\text{-}CoA}$, the true Ω_{pyr} should be $4.08 + 1.09 = 5.17‰$.

The effect of non-statistical isotope distribution within glucose was also seen in δ_{TIC}. Carbon dioxide is produced by decarboxylation of pyruvate and should retain the isotopic composition of positions C-3 and C-4 of glucose, being 2.18‰ enriched in ^{13}C relative to the other positions. Therefore, the isotopic composition of TIC should reflect that of C-3 and C-4 of glucose in those experiments, in which only small amounts of lactate were formed and the CO_2 was not further converted to formate. In pure cultures with glucose at 15 °C, in which lactate and formate formation was very low (Table 1), δ_{TIC} indicates slight ^{13}C enrichment at C-3 and C-4 of glucose (Table 2). On the other hand, $\delta_{ac\text{-}CoA}$ was slightly depleted in ^{13}C with respect to initial δ_{glu} (Table 2). This is in agreement with the relative ^{13}C depletion of $\delta_{C1,2,5,6}$ in glucose. Stronger production of formate and lactate in the pure culture on glucose at 30 °C resulted, as expected, in a decrease of $\alpha_{sacc/TIC}$ because of the fractionation at the branch point pyruvate and the incomplete conversion of TIC to formate.

At the second branch point acetyl-CoA (Fig. 1), carbon flow splits to the formation of acetate (via acetyl-phosphate), ethanol (via acetaldehyde), and biomass. Ethanol was generally ^{13}C-depleted, resulting in a relatively high $\Omega_{ac\text{-}CoA}$, which was consistently determined by both the vertical difference and the slopes of the regression lines of $\Delta\delta_{etoh}$ and $\Delta\delta_{ac}$ (Fig. 3).

Acetyl-CoA as well as pyruvate is also a precursor of biomass. The carbon flow to biomass at these two branch points was not quantified in this study, so that the relative significance of isotopic change caused by this additional branch could not be considered. Moreover, knowledge on fractionation to the different cell components is limited. For fungi grown on different sugars slight fractionation against ^{12}C was found during the synthesis of amino acids (Abraham and Hesse, 2003), but no research of this kind has been done in bacteria. Fatty acids exhibit in general a slight depletion in ^{13}C with respect to the substrate (Abraham et al., 1998). Since the $\alpha_{sacc/biom}$ observed in our experiments was only small and since in anaerobic microorganisms the carbon flow into anabolic versus catabolic reactions is also relatively small, the error introduced

by ignoring biomass formation for calculation of Ω_{pyr} and $\Omega_{ac\text{-}CoA}$ should be small.

Hydrogen formate lyase reversibly catalyzes the conversion of $H_2 + CO_2$ to formate. The formation of formate observed in our experiments was probably caused by CO_2 reduction. The reaction of $H_2 + CO_2$ to formate is not quantitative, so that CO_2 was still present. Since ^{12}C is generally preferred in reactions with primary isotope effects, the direction, in which the reaction occurs, could be confirmed by the following observations. Formation of formate was increased at 30 °C, and well correlated with a slight decrease in $\alpha_{sacc/TIC}$ (Tables 1 and 2). In chemostat cultures at high p_{H2}, δ_{CO_2} increased with time until a stable value was reached (Fig. 5). There, the non-flushed headspace created a non-steady-state situation for gaseous CO_2, while all dissolved fermentation products were at steady-state. Due to the preferential formation of formate from $^{12}CO_2$, CO_2 in the headspace became enriched in ^{13}C. Isotopic equilibrium of the reversible reaction was not established before 180 changes in the culture volume.

4.3. Carbon flow determines isotope fractionation to the products

In our experiments we found that variations in temperature, type of substrate, and initial substrate concentration did not directly influence the carbon isotope fractionation (Table 1), but resulted in strong shifts in the carbon flow at the branch points, which in turn affected the isotopic composition of the products. The metabolic flexibility of *C. papyrosolvens* allowed these relative changes in carbon flow, so that the isotopic composition of the products varied to some extent, although the fractionation factors to the products at the branch points were constant (Hayes, 2001).

Growth rate is often considered as an important variable affecting the intrinsic isotope fractionation of an organism (Henn et al., 2002; Londry and DesMarais, 2003). However, little is known about the effect of growth rate, since it is difficult to control in batch culture. Here, using chemostat cultures, fractionation by *C. papyrosolvens* was found to be independent of growth rate in the range investigated (Fig. 4). An effect would actually be expected only if a change of the metabolic pathway was associated with a change in growth rate, or if different enzymes (isoenzymes) catalyzed the same reactions at the different growth rates and had different fractionation characteristics. From our experiments we conclude that if there were isoenzymes involved, they did not differ in their discrimination of carbon isotopes.

We also tested the influence of the p_{H2}, e.g., by running chemostat cultures at high and low p_{H2} (Fig. 4; Table 2) and by growing co-cultures with H_2-consuming methanogens (Fig. 2; Table 1). The H_2 partial pressure had a strong effect on carbon flow in *C. papyrosolvens* and thus also on carbon isotope fractionation. The strong effect of p_{H2} is a general feature for carbon flow in fermenting microorganisms and anoxic environments (Zeikus, 1983; Dolfing, 1988; Stams, 1994; Schink, 1997). In *C. papyrosolvens*, pyruvate is decarboxylated by pyruvate-ferredoxin oxidoreductase. During this reaction ferredoxin is reduced, which has a very low redox potential ($E^{o\prime} = -0.41$ V) that allows the formation of hydrogen even at high p_{H2} (Gottschalk, 1986). Our experiments confirmed that formation of H_2 occurred up to very high partial pressures as expected. Furthermore, we observed an increase in acetate formation at low p_{H2}. This change is only slightly expressed in the different chemostat setups, but in the co-cultures growing on cellulose acetate was the almost exclusive product as soon as a low p_{H2} was reached. Removal of H_2 thus channeled the carbon flow into acetate, which is a preferred fermentation product, since it allows generation of additional ATP by substrate chain phosphorylation (Gottschalk, 1986). It is well known from various culture experiments that removal of H_2 results in preferential production of acetate (Zeikus, 1983; Dolfing, 1988; Stams, 1994; Schink, 1997).

A low p_{H2} is also characteristic for natural conditions in anoxic environments. This characteristic allows us to narrow down the isotopic composition of acetate produced by primary fermentation in the environment. Considering the carbon flow at the two branch points (Fig. 1), very low amounts of lactate should be produced, yielding acetyl-CoA isotopically similar to pyruvate, so that the $\delta^{13}C$ of the produced acetate will almost exclusively be determined by the branching of carbon flow at acetyl-CoA. There, p_{H2} determines f_{ac} and consequently δ_{ac} (p_{H2} included in Fig. 3 for *C. papyrosolvens* grown on cellulose). Since hydrogen is only present at very low amounts in most anoxic soils and sediments, we may expect a high f_{ac} (>0.85). Consequently, δ_{ac} should be only slightly enriched (<3‰) in ^{13}C with respect to the substrate used in fermentation, e.g., polysaccharide.

Our study addressed the fractionation of stable carbon isotopes during anaerobic fermentation of different saccharides using *C. papyrosolvens* as model organism. Though this bacterium is a typical fermenting bacterium in anoxic rice field soil and other outdoor environments, other fermenting microorganisms with different metabolic pathways may be more important in other environments. Therefore, additional work on other types of microorganisms, e.g., propionate-producing fermenting bacteria, is required. Beside the fractionation factors to the individual fermentation products, we were able to elucidate the fractionation patterns at the metabolic branch points of *C. papyrosolvens*. These branch points are also part of metabolic pathways of other groups of microorganisms, so that the fractionation observed for *C. papyrosolvens* should allow predicting carbon isotope fractionation in other organisms.

4.4. Biogeochemical implications

Our results contribute to the understanding of the biogeochemical cycling of $^{13}C/^{12}C$ in carbohydrates, acetate, lactate and ethanol in anoxic environments. Acetate is one of the most important intermediates in anoxic soils

and sediments. It is the precursor of 25–50% and 60–70% of methane in organic rich marine and freshwater sediments, respectively (Blair and Carter, 1992). For modeling of isotopic fractionation by acetate-consuming or producing processes in anoxic habitats δ_{ac} must be known. For example, δ_{ac} may be used for determining the contribution of acetoclastic and hydrogenotrophic methanogenesis to methane production (Conrad, 2005) or the relative consumption of acetate in sulfate reduction versus methanogenesis (Blair and Carter, 1992). Despite the importance of δ_{ac}, it can hardly be measured directly in natural environments, since acetate-consuming reactions, like sulfate reduction or acetoclastic methanogenesis, decrease acetate concentrations to steady-state values that are usually too low for analysis. Therefore information on the isotope ratio of acetate in natural environments is generally rare. Only a limited number of studies measured δ_{ac} in the field (Blair et al., 1987; Blair and Carter, 1992) or in incubations (Sugimoto and Wada, 1993; Conrad et al., 2002). Calculation of δ_{ac} from the $\delta^{13}C$ value of total organic carbon (δ_{TOC}) would overcome these analytical problems, if the isotope fractionation during fermentation of TOC is known. Our study presents experimental evidence for only a slight (<3‰) ^{13}C enrichment of acetate with respect of δ_{TOC} at low p_{H2}, which is typical for natural environments at steady-state. This confirms earlier assumptions (Gelwicks et al., 1989; Blair and Carter, 1992), now allowing the estimation of δ_{ac} from δ_{TOC} with more confidence.

TOC is the ultimate source of primary fermentation products like acetate and ethanol. The degradable part of TOC mostly consists of polysaccharides. For example, plant material, such as rice straw, is composed of 32–37% cellulose and 29–37% hemicellulose, which are relatively easily degradable, and only 5–15% lignin, which is hardly degraded under anoxic conditions (Tsutsuki and Ponnamperuma, 1987; Watanabe et al., 1993; Shen et al., 1998). We found that the isotope ratio of the fermentation products of cellulose did not change during the course of fermentation. Similarly, δ_{TOC} was stable with depth in the sediment of Cape Lookout Bight, although the TOC content decreased by up to 30% (Blair and Carter, 1992). The lack of isotope discrimination during the degradation of cellulose in anoxic soils and sediments is probably due to the fact that hydrolysis of polysaccharides is usually the rate-limiting step (Billen, 1982), so that the hydrolysis products (e.g., glucose and cellobiose) are metabolized quantitatively without an isotope effect. However, during fermentation of soluble carbohydrates (e.g., glucose and cellobiose) the isotopically lighter molecules are preferentially selected, as shown in the present study. In summary, we can assume that δ_{TOC} in the environment stays unchanged during its degradation, if it consists primarily of polysaccharides. Then, it is straightforward to calculate the isotope ratio of fermentation products using experimentally determined fractionation data, such as provided in our study. The δ_{TOC} in sediments and soils is originally that of the TOC of dead plants and algae. δ_{TOC} derived from C_3 plants usually has values around −28‰, whereas from C_4 plants it is about −14‰ (O'Leary, 1988). In this study we found that the isotope fractionation during fermentation to acetate was the same both for saccharides derived from C_3 and C_4 plants, as the ^{13}C isotopic signatures of cellulose and cellobiose were characteristic for C_3 plants and that of glucose for C_4 plants, respectively.

In conclusion, the fractionation characteristics determined for the fermentation of carbohydrates might be useful to calculate from δ_{TOC} the isotopic signatures of the fermentation products, if these cannot be determined directly, and thus help constraining biogeochemical models of C-flux in anoxic environments, such as wetland soils, freshwater and ocean sediments. As an immediate result, we propose to use δ_{TOC} (+0 to 3‰) as proxy for δ_{ac} if the latter cannot be measured directly. We would like to emphasize, however, that this proposal should be tested rigorously by measurements in natural environments as it is presently based on culture experiments only.

In addition, fermentation processes in which products other than acetate are formed may dominate other environments. Compared to acetate, fermentation products such as lactate or ethanol are usually considered to be of less importance in natural environments. However, recent studies indicate that ethanol may be an important intermediate in methanogenic peat mires (Horn et al., 2003; Metje and Frenzel, 2005), which cover a large area in the Northern Latitudes and play an important role in the global methane budget (Harriss et al., 1993). According to our results, a high carbon flow to ethanol would result in production of acetate that is relatively strongly (<17‰) enriched in ^{13}C compared to TOC. Hence, our results might be relevant for interpreting carbon flow and isotope discrimination in anoxic peat.

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