New step in anaerobic digestion modeling: estimating changes in isotopic composition as a way to reveal metabolic pathways

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Abstract
Introducing the fractions of heavy isotope in products, substrate and biomass into the traditional anaerobic digestion models it was possible to reveal metabolic pathways of substrate transformation by microbial communities. In this paper, as an example, evolution of $^{13}C/C$ and $\delta^{13}C$ in methane and carbon dioxide during labelled and natural acetate methanization in the parallel batch experiments under the different initial ammonium concentrations, previously carried out by Grossin-Debattista (2011), was mathematically described based on stoichiometric chemical reactions, microbial dynamics and equations for the isotope ^{13}C accumulation in products as well as its redistribution between substrate and products. Isotope fractionation of ^{13}C and ^{12}C was taken into account to estimate evolution of $\delta^{13}C$. First time, the same approach was used to describe substrate methanization using labelled and natural substrate in the parallel batch experiments. The model of batch reactors showed that an input of acetoclastic and non-acetoclastic methanogenesis depended on a ratio between the initial concentrations of *Methanosarcinaceae*, *Methanosaetaceae* and acetate-oxidizers. At the high ammonium concentration the input of syntrophic acetate oxidation was developing in time and becoming dominant during incubation.

Keywords
Labelled and natural acetate methanization, ammonium, acetoclastic and non-acetoclastic methanogenesis, kinetic isotope effect

INTRODUCTION

AD is a multi-step process of series and parallel reactions in which several key groups of bacteria take part. Since 2002, a joint effort to create a unified language and to propose a general structured model has resulted in the generic anaerobic digestion model ADM1 (Batstone et al 2002). The ADM1 is based on the consumption and production rates of the measured chemical components not considering changes of isotopic composition. In contrast to ADM1, in the new approach redistribution of isotopic composition of substrate, product and biomass was taken into account. As a result, changes in isotopic composition due to substrate consumption, biomass growth and isotope fractionation have been used to reveal metabolic pathways of acetate transformation by anaerobic microbial communities at the different initial ammonium concentration. Earlier, syntrophic acetate oxidation at the high ammonia concentration was demonstrated in a number of works (e.g. Schnurer et al., 1994).

Traditionally, to describe changes in isotope composition the approach based on equilibrium fractionation equations or steady-state kinetic fractionation equations was used and it can only be applicable when the substrate concentration is high enough (Thullner et al., 2008). In our approach, we used a common mixed-culture kinetic biological model coupled to stoichiometric fractionation equations. Instead of using the concentrations of each isotopologues (^{12}C and ^{13}C), total concentrations and isotope ratios were considered as variables.

GROSSIN-DEBATTISTA's EXPERIMENTS

The detailed experimental procedure was described earlier (Grossin-Debattista, 2011). Mesophilic acetate methanization at the different ammonium concentrations was carried out at 35.0°C. Acetate labelled by ^{13}C in the methyl group ($^{13}CH_3COOH$) was used in the single batch test wherein natural acetate was used in the duplicate batch tests. In accordance with automated ribosomal intergenic spacer analysis (ARISA) and fluorescent in situ hybridization (FISH), the most presented populations in inoculum were *Methanosarcinaceae* and *Methanosaetaceae*. Using stable carbon isotope signatures of $\delta^{13}CH_4$ and $\delta^{13}CO_2$ it was shown that at the low ammonium concentration the acetoclastic pathway of acetate conversion to methane was dominated and performed by *Methanosarcinaceae* and

Methanosaetaceae. At the high ammonium concentration it was supposed that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis probably occurred.

FOUR-SPECIES MODEL

The following equations were considered:

$$\frac{d\,\mathrm{CH_3COOH}}{d\,t} = -\rho_{m\,Sar}\,B_{Sar}\,\frac{\mathrm{CH_3COOH}}{K_{Sar}+\mathrm{CH_3COOH}} - \rho_{m\,Sae}\,B_{Sae}\,\frac{\mathrm{CH_3COOH}}{K_{Sae}+\mathrm{CH_3COOH}} - \rho_{m\,AcOx}B_{AcOx}\,\frac{\mathrm{CH_3COOH}}{K_{AcOx}+\mathrm{CH_3COOH}}$$

$$\frac{d\,\mathrm{CH_4}}{d\,t} = Vol*\{(1-Y_{Sar})\rho_{m\,Sar}\,B_{Sar}\,\frac{\mathrm{CH_3COOH}}{K_{Sar}+\mathrm{CH_3COOH}} + (1-Y_{Sae})\rho_{m\,Sae}\,B_{Sae}\,\frac{\mathrm{CH_3COOH}}{K_{Sae}+\mathrm{CH_3COOH}}$$
$$+ 0.25\,\rho_{mH2/H2CO3}\,B_{hydr}\,\frac{\mathrm{H_2}}{K_{H2}+\mathrm{H_2}}\,\frac{\mathrm{H_2CO_3}}{K_{H2CO3}+\mathrm{H_2CO_3}}\}$$

$$\frac{d\,\mathrm{H_2CO_3}}{d\,t} = (1-Y_{Sar})\rho_{m\,Sar}B_{Sar}\,\frac{\mathrm{CH_3COOH}}{K_{Sar}+\mathrm{CH_3COOH}} + (1-Y_{Sae})\rho_{m\,Sae}B_{Sae}\,\frac{\mathrm{CH_3COOH}}{K_{Sae}+\mathrm{CH_3COOH}}$$
$$+ 2(1-Y_{AcOx})\rho_{m\,AcOx}B_{AcOx}\,\frac{\mathrm{CH_3COOH}}{K_{AcOx}+\mathrm{CH_3COOH}} - 0.25\rho_{mH2/H2CO3}\,B_{hydr}\,\frac{\mathrm{H_2}}{K_{H2}+\mathrm{H_2}}\,\frac{\mathrm{H_2CO_3}}{K_{H2CO3}+\mathrm{H_2CO_3}}$$
$$- K_C(\mathrm{H_2CO_3}-\mathrm{H_2CO_{3sat}}) \qquad (1)$$

$$\frac{d\,\mathrm{H_2}}{d\,t} = 4(1-Y_{AcOx})\rho_{m\,AcOx}B_{AcOx}\,\frac{\mathrm{CH_3COOH}}{K_{AcOx}+\mathrm{CH_3COOH}} - \rho_{mH2/H2CO3}\,B_{hydr}\,\frac{\mathrm{H_2}}{K_{H2}+\mathrm{H_2}}\,\frac{\mathrm{H_2CO_3}}{K_{H2CO3}+\mathrm{H_2CO_3}}$$

$$\frac{d\,B_{Sar}}{d\,t} = Y_{Sar}\rho_{m\,Sar}\,B_{Sar}\,\frac{\mathrm{CH_3COOH}}{K_{Sar}+\mathrm{CH_3COOH}} - k_{d\,Sar}B_{Sar}$$

$$\frac{d\,B_{Sae}}{d\,t} = Y_{Sae}\rho_{m\,Sae}\,B_{Sae}\,\frac{\mathrm{CH_3COOH}}{K_{S\,sae}+\mathrm{CH_3COOH}} - k_{d\,Sae}B_{Sae}$$

$$\frac{d\,B_{AcOx}}{d\,t} = Y_{AcOx}\rho_{m\,AcOx}\,B_{AcOx}\,\frac{\mathrm{CH_3COOH}}{K_{AcOx}+\mathrm{CH_3COOH}} - k_{d\,AcOx}B_{AcOx}$$

$$\frac{d\,B_{hydr}}{d\,t} = Y_{hydr}\rho_{mH2/H2CO3}\,B_{hydr}\,\frac{\mathrm{H_2}}{K_{H2}+\mathrm{H_2}}\,\frac{\mathrm{H_2CO_3}}{K_{H2CO3}+\mathrm{H_2CO_3}} - k_{d\,hydr}B_{hydr}$$

where $\mathrm{CH_3COOH}$, $\mathrm{H_2}$ and $\mathrm{H_2CO_3}$ are the acetate, hydrogen and carbonic acid concentrations; B_{Sar}, B_{Sae}, B_{AcOx}, B_{hydr} are the concentration of acetoclastic methanogens *Methanosarcinaceae* and *Methanosaetaceae*, acetate-oxidizers and hydrogenotrophic methanogens; $\mathrm{CH_4}$ is the total methane quantity; $\rho_{m\,Sar}$, $\rho_{m\,Sae}$, $\rho_{m\,AcOx}$, $\rho_{mH2/H2CO3}$ are the maximum specific rate of acetate utilization by different microorganisms; K_{Sar}, K_{Sae}, K_{AcOx}, K_{H2CO3}, K_{H2} are the corresponding half-saturation constants; $k_{d\,Sar}, k_{d\,Sae}$, $k_{d\,AcOx}, k_{d\,hydr}$ are the corresponding specific biomass decay coefficients; Y_{Sar}, Y_{Sae}, Y_{AcOx}, $Y_{H2/H2CO3}$ are the corresponding biomass yield coefficients; K_C is the mass transfer coefficient; $\mathrm{H_2CO_{3sat}}$ is the saturated value of $\mathrm{H_2CO_3}$; Vol is the liquid volume. Moles units were used in the four-species model.

Introducing the fractions of $^{13}\mathrm{C}$ in products as $f^P = {}^{13\mathrm{C}}P/P$ the following equation was used to describe the dynamics of $^{13}\mathrm{C}$ in products:

$$\frac{df^P}{dt} = \frac{1}{P}\,\frac{d\,{}^{13\mathrm{C}}P}{dt} - \frac{{}^{13\mathrm{C}}P}{P^2}\,\frac{dP}{dt} = \frac{dP/dt}{P}\left\{\frac{d\,{}^{13\mathrm{C}}P/dt}{dP/dt} - f^P\right\} \approx \frac{dP/dt}{P}\left(\frac{1}{\alpha}\,f^S - f^P\right) \qquad (2)$$

where $f^S = {}^{13C}S/S$ is the fraction of ^{13}C in substrate; dP/dt is the production rate of P; α is the carbon fractionation factor. Similar equations were written for biomasses. To describe isotope ^{13}C enrichment in substrate**,** a mass balance equation for total concentration of ^{13}C in substrate (S), products (P_j) and biomass (B) should be considered:

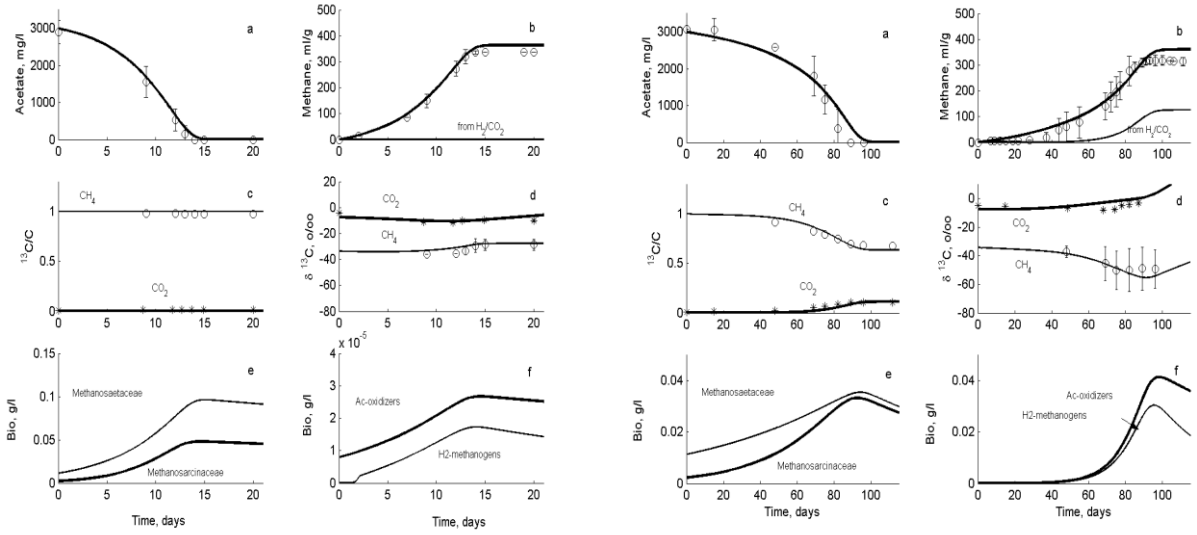
$$S^{13C} + P^{13C} + B^{13C} = f_C^S S + \sum_j f_C^{P_j} P_j + \sum_i f_C^{B_i} B_i = const \quad (3)$$

The following equation can be written from the equation (3):

$$\frac{df_C^S}{dt} = -\frac{1}{S}\left[\frac{dS}{dt} f_C^S + \sum_j \frac{df_C^{P_j}}{dt} P_j + \sum_j \frac{dP_j}{dt} f_C^{P_j} + \sum_i \frac{df_C^{B_i}}{dt} B_i + \sum_i \frac{dB_i}{dt} f_C^{B_i} \right] \quad (4)$$

In the earlier paper (Vavilin, 2013), it was assumed that the fractionation is maximal at high substrate concentration and it drops to zero when the substrate is exhausted, thus the right parts of equations (4) were multiplied on the empirical function $\phi = \frac{(S/K)^n}{1+(S/K)^n}$, where K is the coefficient reflecting the decrease in isotopic fractionation with decreasing concentration of substrate; $n \geq 1$. In case of the totally labeled acetate, $\alpha = 1$, $f^{CH3COOH} = 1$ and $f^{H2CO3} = 0$ were used for acetoclastic methanogenesis and $f^{CH3COOH} = 0.5$ was used for acetate oxidation, respectively. Because carbonic acid was the product in acetoclastic methanogenesis and acetate oxidation but it was the substrate in hydrogenotrophic methanogenesis, to describe $\delta^{13}CO_2$ both inputs were considered. It was assumed that the value of $\delta^{13}C$ in carbonic acid and carbon dioxide were the same. The same initial values of biomass concentration

Fig. 1 Fig.2



Figures 1 and 2. Time profiles of the system variables during labeled and natural acetate methanization at the low ($0.18\,g\,l^{-1}$**)** and high ($3.6\,g\,l^{-1}$**)** initial ammonium concentrations. Symbols represent the values measured (Grossin-Debattista, 2011) and lines are the model results.

and kinetic parameter values were used in the model for labelled and natural acetate methanization. For all microorganisms it was assumed that the biomass yield coefficients were the same and equal to $0.025\,mM/\,mM$. In all simulations, the values of $K_C = 0.0025\,d^{-1}$ and $H_2CO_{3\,sat} = 0.1M$ were used as the mass transfer coefficients for CO_2. The values of $n = 2$, $K_{CH3COOH} = 0.08\,M$ and $K_{H2CO3} = 0.037\,M$ were used in the function ϕ. In the model, a dissociation of carbonic acid was not considered. The stable carbon isotope signature ($\delta^{13}C$) was expressed in ‰ deviations of a sample from Pee Dee Belemnite (PDB) carbonate as the standard: $^{13}C/^{12}C = 0.0112372$.

RESULTS AND DISCUSSION

Figure 1 demonstrates system dynamics in the parallel reactors with label and natural acetate at the low ($0.18\,g\,l^{-1}$) initial ammonium concentrations. In accordance with the model, during all incubation time acetoclastic methanogenesis dominated with *Methanosaetaceae* and *Methanosarcinaceae* competing for acetate. A participation of *Methanosaetaceae* and *Methanosarcinaceae* was shown experimentally by Grossin-Debattista (2011) using FISH and ARISA methods. In the initial period, the concentration of *Methanosaetaceae* was significantly higher than that of *Methanosarcinaceae* but they became comparable at the end of batch experiments (see Fig. 1e). The corresponding specific biomass growth rates μ_m and half-saturation coefficients K_S were of $0.38\,d^{-1}$ and $0.017\,M$ for *Methanosarcinaceae,* $0.19\,d^{-1}$ and $0.0033\,M$ for *Methanosaetaceae*, and $0.125\,d^{-1}$ and $0.0066\,M$ for acetate-oxidizers, respectively. For hydrogenotrophic methanogens, in agreement with the literature values (Batstone et al., 2002), maximum specific growth rate of biomass μ_m were appointed to be high enough. During all incubation time the concentrations of acetate-oxidizers and hydrogenotrophic methanogens were negligible (Fig. 1f). Due to labeling the methyl group in acetate all heavy isotope ^{13}C transferred directly to methane (Fig. 1c). Lower fractionation coefficient $\alpha^{CH3COOH}$ was assumed for *Methanosaetaceae* (1.009) than that one for *Methanosarcinaceae* (1.025) which was in accordance with Goevert and Conrad (2009). The model showed that the current value of $\delta^{13}CH_4$ (Fig. 1d) was dependent on a ratio between the rates of acetoclastic methanogenesis performing by *Methanosaetaceae* and *Methanosarcinaceae*.

Figure 2 demonstrates system dynamics at the high ($3.6\,g\,l^{-1}$) initial ammonium concentrations. The corresponding specific biomass growth rates μ_m at the high ammonium concentration were of 0.06 and $0.0025\,d^{-1}$ for *Methanosarcinaceae* and *Methanosaetaceae*, respectively, but the growth rates of acetate-oxidizers and hydrogenotrophic methanogens (ammonium tolerant) were assumed the same as these values at the low ammonium concentration. Heavy isotope ^{13}C transferred not only to methane but to carbon dioxide/carbonic acid during acetoclastic and non-acetoclastic methanogenesis. Methanogenesis from H_2/CO_2 results in significantly lower $\delta^{13}CH_4$ values than that of acetoclastic metabolism, which is reflected in a value of the corresponding fractionation coefficient $\alpha^{CO2} = 1.075$. In accordance with the model, acetoclastic- and non-acetoclastic methanogenesis were developing since the start of incubation (Figs. 2e and 2f). A dominance of non-acetoclastic methanogeneses occurred only at the end of incubation. Figures 1 and 2 evidenced that the changes of $\delta^{13}C$ in carbon dioxide were much less than those in methane. Comparatively to the experimental data, the model showed a little lower methane production at the final stage because liquid samples were taken for analysis during the long-time experiments.

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