APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1986, p. 180–187
0099-2240/86/010180-08$02.00/0

Vol. 51, No. 1

Microbial Ecophysiology of Whey Biomethanation: Intermediary Metabolism of Lactose Degradation in Continuous Culture

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Received 27 June 1985/Accepted 11 October 1985

The intermediary carbon and electron flow routes for lactose degradation during whey biomethanation were studied in continuous culture. The chemostat was operated under lactose-limited conditions with a 100-h retention time. The carbon balance observed for lactose degradation was 4.65 mmol of CH_4, 4.36 mmol of CO_2 and 1.15 mmol of cellular carbon per mmol of lactose consumed, with other intermediary metabolites (i.e., acetate, lactate, etc.) accounting for less than 2% of the lactose consumed. The carbon and electron recoveries for this biomethanation were 87 and 90%, respectively. ^{14}C tracer studies demonstrated that lactose biomethanation occurred in three distinct but simultaneous phases. Lactose was metabolized primarily into lactate, ethanol, acetate, formate, and carbon dioxide. During this hydrolytic phase, 82% of the lactose was transformed into lactate. These metabolites were transformed into acetate and H_2-CO_2 in a second, acetogenic, phase. Finally, the direct methane precursors were transformed during the methanogenic phase, with acetate accounting for 81% of the methane formed. A general scheme is proposed for the exact carbon and electron flow route during lactose biomethanation, which predicts the prevalent microbial populations in this ecosystem.

Whey is an important by-product of the cheese industry. The production of 1 lb (0.45 kg) of cheese generates between 6 (2.7 kg) and 10 (4.5 kg) lb of whey, with an annual production in the United States estimated at 36×10^9 lb (16.2×10^9 kg) (C. J. Clanton, P. R. Goodrich, P. A. Lee, and B. D. Backus, Meet. Am. Soc. Agric. Eng., paper 81-6007, 1981). Whey is composed of 93% water and 7% solids. The solids are composed of lactose, which represents more than 70%, proteins, which account for 15%, and lipids and various salts accounting for the balance of solids (8). A new process of ultrafiltration and reverse osmosis allows the recovery of the valuable protein portion in whey, and the remaining solute is mainly lactose (A. A. Texiera, D. E. Johnson, and R. A. Zale, Meet. Am. Soc. Agric. Eng., paper 82-6510, 1982). Whey is a major problem for the cheese industry because it is a heavy pollutant with a high biological oxygen demand of approximately 40,000 mg/liter, and its discharge into sewage treatment plants can cause serious problems of overloading (Clanton et al., Meet. Am. Soc. Agric. Eng., 1981). Several different but expensive and energy-requiring systems have been designed to dispose of whey in lieu of biomethanation, including fermentation to alcohol (6) and spray drying and utilization as a feed stock (8). Anaerobic digestion of whey has already proven to be feasible based on parameters such as chemical oxygen demand and total solids reduction. The calculated efficiency of the pollution reduction during different studies shows a range of chemical oxygen demand removal between 60 and 80% (Clanton et al., Meet. Am. Soc. Agric. Eng., 1981).

Studies on the intermediary metabolism during anaerobic degradation of organic matter, including identification of the intermediary metabolites involved in carbon and electron flow, have been performed in diverse anoxic environments (4, 5, 7, 10–13, 16, 20, 21, 24; T. Cohen, Ph.D. thesis, University of Amsterdam, The Netherlands, 1982). During anaerobic digestion, complex organic matter is hydrolyzed into acids, such as acetate, lactate, propionate, and butyrate, and neutral products, such as ethanol and H_2-CO_2, which are detected as intermediary metabolites (4, 5, 7, 11, 21). The long-chain fatty acids and neutral products are further degraded to acetate, H_2, and CO_2 (12; Cohen, Ph.D. thesis). Acetate is usually the major detectable intermediary metabolite during anaerobic digestion and is the direct precursor for about 70% of the total methane formed, with the balance principally derived from carbon dioxide reduction (10, 20, 23). Engineering studies on anaerobic digestion of whey in an up-flow reactor reported acetate, lactate, propionate, and butyrate as detectable intermediary metabolites of whey biomethanation (S. T. Yang, M. R. Okos, and J. C. Nye, Meet. Am. Soc. Agric. Eng., paper 82-374, 1982).

To a limited extent, ^{14}C-radioactive molecules have been used to determine the path of carbon and electron flow during the anaerobic digestion of specific organic matter in diverse ecosystems (12, 13; Cohen, Ph.D. thesis). This radioisotopic technique has been used to determine degradation patterns for sugars, organic acids, and alcohols in waste digestors (Cohen, Ph.D. thesis). To our knowledge, such a technique has not been used to determine the path of lactose during biomethanation of whey.

The general purpose of the present study was threefold. The first was to develop a model system for continuous processing of whey into methane under steady-state, lactose-limited conditions. The second was to quantify the fermentation balance and the type of intermediary metabolites (i.e., volatile fatty acids, alcohols, and gases). The last was to determine by ^{14}C tracer studies the intermediary metabolic reactions for lactose transformation into methane and carbon dioxide.

MATERIALS AND METHODS

Chemicals, gases, and isotopes. All chemicals used were of reagent grade or better and were obtained from Sigma Chemical Co., St. Louis, Mo., or Mallinckrodt, Inc., Paris, Ky. All gases used were obtained from Matheson Scientific, Inc., Joliet, Ill. All radioactive agents were obtained from

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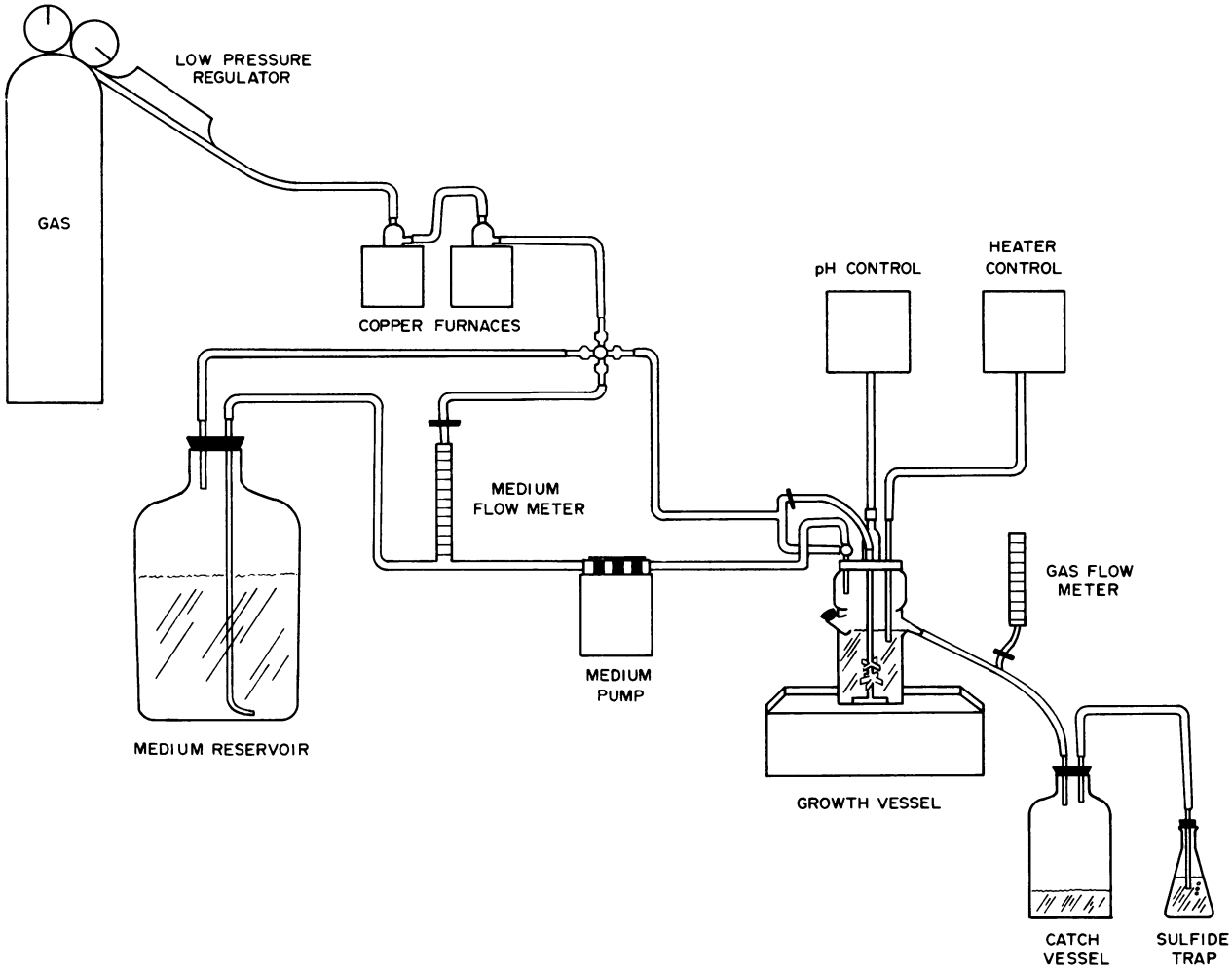

FIG. 1. Schematic of the whey-processing chemostat system.

New England Nuclear Corp., Boston, Mass.). The specific-labeled substrates were: [U-^{14}C]lactose (701 mCi/mmol), [1-^{14}C]acetate (1.8 mCi/mmol), [2-^{14}C]acetate (54 mCi/mmol), [U-^{14}C]acetate (54.7 mCi/mmol), [^{14}C]formate (55 mCi/mmol), [U-^{14}C]lactate (40 mCi/mmol), [^{14}C]sodium bicarbonate (0.25 mCi, 2.7 mg in 2.5 ml of H_2O). When the radioisotopes were in ethanol, ethanol was evaporated by gassing it with nitrogen, and the ^{14}C-labeled substrate was dissolved in sterile double-distilled water.

Chemostat system. Figure 1 illustrates the chemostat used in the present study. A New Brunswick Multigen continuous culture fermentation system (New Brunswick Scientific Co., Inc., Edison, N.J.) was modified for the present study. The vessel of 1-liter capacity, with a 260-ml working volume, has a short side arm sealed with a black butyl rubber stopper to allow gas and liquid sampling. The pH was monitored by means of a combination electrode (Ingold Electronic, Inc., Andover, Mass.) and a New Brunswick pH controller and maintained at 7.1 ± 0.1 by addition of sterile 3 N NaOH via a peristaltic pump (Sigmamotor, Inc., Middleport, N.Y.). The temperature was maintained at 37 ± 1°C with a recirculating water bath (Hacke, Berlin, Federal Republic of Germany). The sterile feeding solution was injected with a peristaltic pump (Gilson Medical Electronics, Middleton, Wis.). The retention time was accurately maintained at 100 h. The chemostat was stirred at 200 rpm, and the headspace was continuously gassed with nitrogen at 12 cm^3/min. Traces of oxygen in the nitrogen gas were removed by passing through reduced copper filings in furnaces heated at 250°C (Sargent Welch Scientific Co., Skokie, Ill.). The gas was sterilized by passing through a sterile glass wool filter. The spent medium was collected in a sterile carboy. The off gases were passed through a saturated solution of zinc acetate before being vented. Prior to any use, the vessel, tubing, and solutions were autoclaved for 40 min at 120°C. All tubing used was either stainless steel or black butyl rubber of 0.25-in. (0.64-cm) thickness.

The chemostat medium was made by adding 10 g of dry whey (Lactofrance, France) to 1 liter of phosphate-buffered basal (PBB) medium (17). The pH of the solution was adjusted to pH 7.2 prior to autoclaving. The medium was sterilized in a 3-liter carboy at 120°C for 40 min. Precipitation was noticed after sterilization, and this sometimes caused plugging of the tubing used to dispense the medium after several weeks of operation. When this problem occurred, the complete feeding line was aseptically replaced by a new one. The medium contained (per liter) 7.8 g of lactose, 395 mg of NH_4, 7.5 mg of sulfate, 1,800 mg of potassium, 520 mg

of sodium, 88.3 mg of calcium, and 43.1 mg of magnesium. No nitrite or nitrate was detected.

The inoculum was obtained from a fixed-bed up-flow digestor operated at the Societe Lyonnaise des Eaux, Le Pec, France. A subsample was anaerobically removed and introduced into a 160-ml serum vial (Wheaton Industries, Millville, N.J.) that contained an atmosphere of nitrogen. The bacteria were removed from their support by injecting 20 ml of reduced PBB medium and by shaking the vial for several hours. The suspension containing the detached bacteria was removed with a syringe equipped with an 18-gauge, 1.5-in. (3.8-cm) needle (Becton Dickinson & Co., Rutherford, N.J.). The cell suspension was injected into the chemostat, which contained reduced medium. Growth and methane production were established prior to setting the dilution rate at 100 h by turning on the peristaltic pump. The off gas volume per unit of time was determined by water displacement, and methane and carbon dioxide content were analyzed. The daily production (V) of methane and carbon dioxide was calculated by the following equation: V = micromoles of CH_4 or CO_2 in 1 ml of off gas × volume of off gas per 24 h (in milliliters).

When methane production was constant for more than 2 months, the chemostat was assumed to operate at steady-state conditions. After this adaptation period, reducing agent was no longer added to the medium which fed the chemostat.

Measurement of substrate and metabolic products. The concentrations of CO_2 and CH_4 in the off gas from the chemostat were measured by sampling (0.4 ml) with a 1-ml glass syringe equipped with a pressure lock valve (Mininert Anspec Co., Ann Arbor, Mich.). CO_2 was determined with a gas chromatograph (model 417; Packard Instrument Co., Inc., Rockville, Md.) equipped with a carbosieve-B column (120/140 mesh) (Supelco, Inc., Bellafonte, Pa.) and a thermal conductivity detector. The column was operated at 95°C with helium as the carrier gas at a flow rate of 60 cm^3/min.

Methane was measured with a gas chromatograph (model 660 D Aerograph; Varian Associates, Palo Alto, Calif.) equipped with a Poropack R column (80/100 mesh) (Anspec Co., Warrenville, Ill.) and a flame ionization detector. The column was operated at room temperature with nitrogen as the carrier gas (30 cm^3/min). The amounts of gas were calculated by Bunsen coefficients and Henry's law (1).

The dissolved hydrogen concentration was determined as follows: a 2-ml sample from the chemostat was immediately injected into a 12-ml serum vial (Wheaton Industries) containing an atmosphere of nitrogen and sealed with a rubber butyl stopper. The dissolved hydrogen was extracted from the liquid by shaking the vial for 30 s. A 0.4-ml sample was injected into a gas chromatograph (model 750; GowMac Instruments Co., Bridgewater, N.J.) equipped with a Spherocarb column (GowMac Instruments), with nitrogen as the gas carrier at 25 cm^3/min and operating at 150°C. Hydrogen was measured by the procedures of Thiele et al. (J. Thiele, M. Chartrain, and J. G. Zeikus, unpublished data) with an RGD2 reduction gas detector (Trace Analytical, Menlo Park, Calif.) based on the HgO-to-Hg vapor conver-

TABLE 1. Lactose biomethanation performance parameters of a steady-state whey-processing chemostat[a]

Biomethanation parameters	Value[b]
Lactose consumed	1,420 ± 34
Metabolites detected	
Methane	6,610 ± 360
Carbon dioxide	6,190 ± 450
Cellular carbon	1,640 ± 360
Acetate	41.1 ± 18.72
Propionate	24 ± 3.4
Butyrate	6 ± 4.36
Ethanol	1 ± 0.18
Lactate	16 ± 1.06
Product recovery (%)	
Carbon	87
Electron	90

[a] The chemostat operated with a 100-h retention time and was fed with PBB medium containing 10 g of dry whey per liter. The results presented are the average of values of analyses performed on the chemostat input and output during 1 week.

[b] Values for lactose consumed and metabolites detected are in micromoles per 24 h. Product recovery is percentage.

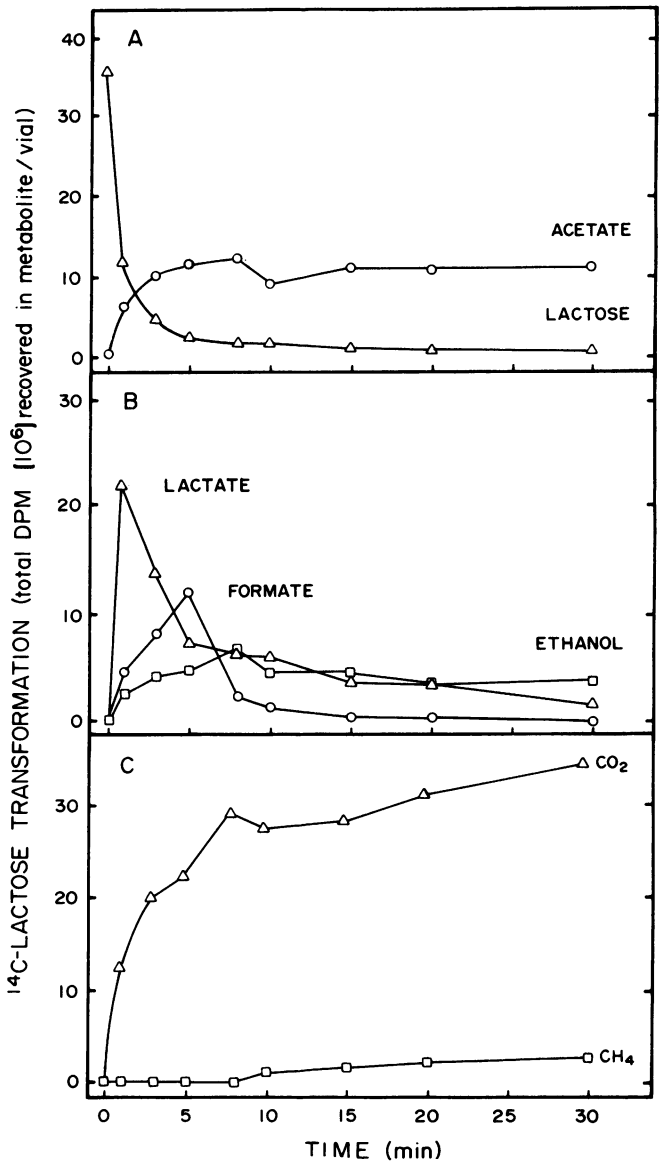

FIG. 2. Fermentation time course for [U-^{14}C]lactose degradation by a whey-processing chemostat sample. The steady-state chemostat operated at a 100-h retention time and was fed with PBB medium containing 10 g of dry whey per liter.

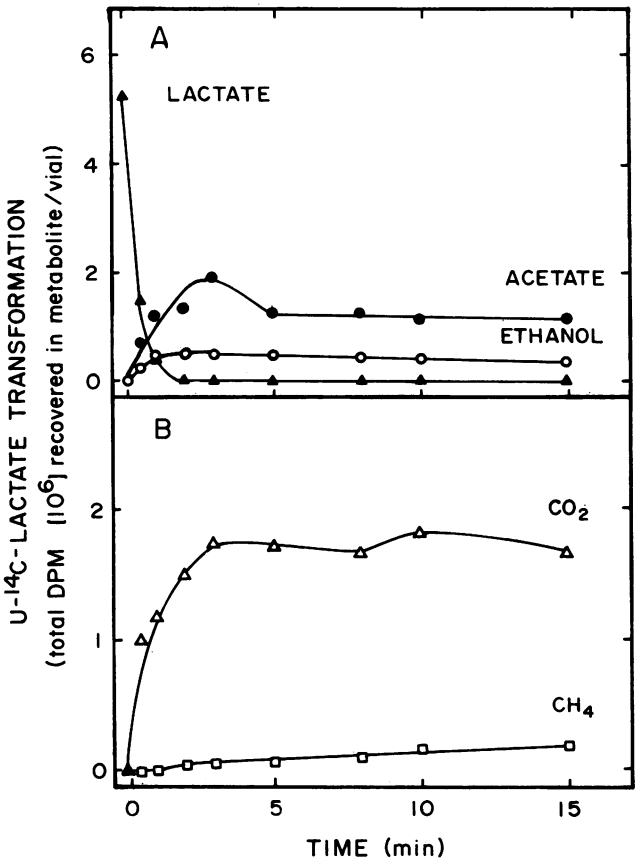

FIG. 3. Fermentation time course for [U-^{14}C]lactate degradation by a whey-processing chemostat sample. The conditions were those described in the legend to Fig. 2.

sion technique (14, 19). The amount of hydrogen was calculated by Bunsen coefficients and Henry's law (1). The background of hydrogen traces present in the vials was determined prior to use and subtracted to obtain the exact value for hydrogen dissolved in the chemostat sample.

Lactose was assayed by enzymatic methods with the supernatant from a sample of the chemostat prepared by centrifugation at 3,000 × *g* for 5 min. The enzymes kit used β-galactosidase and galactose dehydrogenase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and NADH production was measured at 340 nm in a spectrophotometer (model 240: Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

For the determination of alcohols and volatile fatty acids, samples were prepared as follows: 1 ml of a sample from the chemostat was introduced into a 1.5-ml plastic centrifuge tube and acidified with 100 μl of 2 N H_3PO_4 and centrifuged at 3,000 × *g* for 5 min. Ethanol was measured by injecting 2 μl of the supernatant into a Packard gas chromatograph (model 419) equipped with a Super Q (80/100 mesh) column (Alltech Associates, Inc., Deerfield, Ill.) and a flame ionization detector. The column was operated at 180°C, while the detector and injector temperatures were 220 and 200°C, respectively. The carrier gas was helium at 100 cm^3/min. Acetate, propionate, and butyrate were quantified by injecting 2 μl of the supernatant into a Packard gas chromatograph (model 419) equipped with a Chromosorb 101 (80/100 mesh) column (Supelco, Inc.) and a flame ionization detector. The column was operated at 200°C, and the detector and injector temperatures were 240 and 220°C, respectively. Samples for lactate determination were concentrated 10-fold by the following procedure. A large sample from the chemostat (15 to 20 ml) was centrifuged at 3,000 × *g* for 20 min, then 10 ml of the supernatant was adjusted to pH 12 with 3 N NaOH, placed into a 150-ml serum vial sealed with a rubber bung, and frozen by immersion into an acetone-dry ice bath, and the contents were evaporated by freeze-drying. The solid materials were then dissolved in 1 ml of double-distilled water, acidified by injection of 50 μl of HCl, and centrifuged for 5 min at 5,000 rpm. Lactate was analyzed in the supernatant by high-pressure liquid chromatography. A liquid chromatograph (series 3) equipped with a Sigma 10 data station (The Perkin-Elmer Corp., Norwalk, Conn.) and a refractive index detector (Laboratory Data Control, Riviera Beach, Fla.) was used. The sample (100 μl) was injected into a Bio-Rad Aminex Ion-exclusion HPX 87H column fitted with a microguard precolumn (Bio-Rad Laboratories, Richmond, Calif.) operating at room temperature. The solvent system was 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min.

Cell dry weight was determined by filtration of a known volume of sample from the chemostat through a membrane filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) that was dried at 60°C until a constant weight was reached. The amount (in millimoles) of cellular carbon (C) was calculated by the following formula: C = (milligrams [dry weight] of cells × 0.451)/12. The electron composition of the cell was calculated with the value of 4.21 electrons per mole of cellular carbon (9).

Chemical analysis of whey medium. All chemical analysis of the culture medium was performed at the analytical chemistry department of the Societe Lyonnaise des Eaux. Total nitrogen, ammonia, nitrite, and nitrate were measured as described previously (2). Sulfate and sulfide were measured by the method described by Winfrey and Zeikus (22).

Radiotracer experiments. Time-course studies to determine carbon degradation patterns were performed as described below. Samples from the chemostat (10 ml) were removed with a glass syringe with an 18-gauge, 1.5-in. (3.8-cm) needle and immediately injected into an anoxic 60-ml serum vial sealed with a rubber bung and containing a nitrogen atmosphere and 0.1 ml of 2.5% Na_2S. The specific-^{14}C-labeled tracer was then injected with a syringe (Unimetrics; Alltech Associates), and the vial was incubated at 37°C and shaken at 200 rpm/min. [^{14}C]methane and [^{14}C]carbon dioxide were analyzed in gas samples (0.4 ml) by a gas chromatographic-gas proportional counting technique previously described (18). Liquid samples (0.5 ml) were removed by syringe and immediately introduced into a 1.5-ml plastic centrifuge tube containing 50 μl of 2 N H_3PO_4 and frozen in a dry ice and acetone bath to immediately stop the reaction. Prior to analysis, the samples were defrosted and centrifuged for 5 min at 3,000 × *g*. The supernatant (100 μl) was loaded onto a high-pressure liquid chromatography column, and the substrate and intermediary metabolites were separated by the method described above. Every 5 drops were eluted as a fraction in vials with a Gilson FC 100 microfractionator. The vials were filled with 4.5 ml of Instagel scintillation cocktail (Packard Instrument Co.) and counted in a Packard Tricarb scintillation counter. Peaks of radioactivity were detected and identified by comparison with the elution of standards.

The turnover constant of carbon substrates was determined by the following radiotracer method. A sample from the chemostat was removed by the techniques previously described. The samples were introduced by syringe into 60-ml serum vials containing a nitrogen atmosphere. At

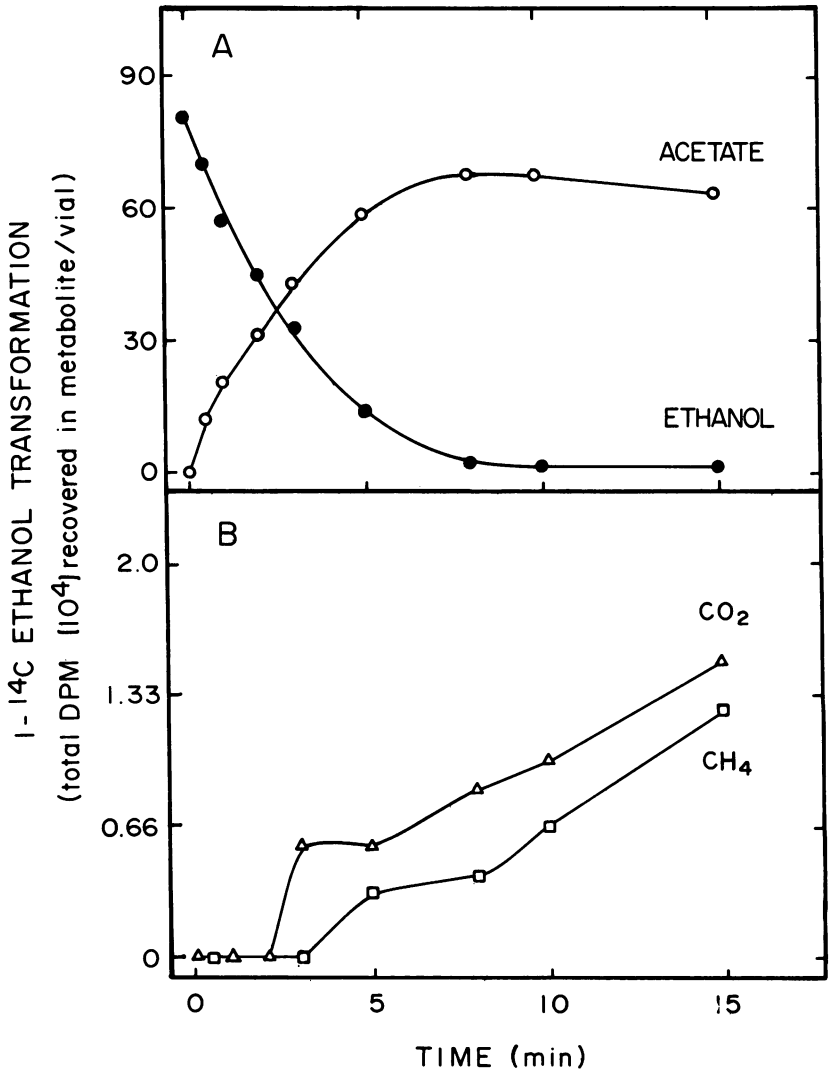

FIG. 4. Fermentation time course for [1-^{14}C]ethanol degradation by a whey-processing chemostat sample. The conditions were those described in the legend to Fig. 2.

regular intervals, subsamples were removed from the vial, and the reaction was stopped as described above. The radioactivity of the specific-^{14}C-labeled added substrate was determined by scintillation counting after separation by the high-pressure liquid chromatography technique as described above. The radioactivity of the fatty acid or alcohol was plotted as $\log_{10}$ versus time, and the turnover constant (K) was calculated from the slope of the graph by the relationship: $K = 2.303 \times \log_{10} (C_0/C_t)$, where C_0 is the initial count and C_t is the count after a given sampling time.

The contribution of lactose to lactate was calculated assuming that a maximum of 4 molecules of lactate can be produced from 1 molecule of lactose and that all the lactate produced was made directly from lactose. When the chemostat was operating with a retention time of 50 h, the input of lactose was 0.45 μmol/h per ml, allowing a maximum production of lactate of $0.45 \times 4 = 1.80$ μmol/h per ml.

The contribution of acetate to methane was determined by the radioisotopic method described by Smith and Mah (20) with [2-^{14}C]acetate. This method assumes that the acetate pool is constant in the chemostat, that the rate of acetate production is equal to the rate of utilization, and that 1 molecule of methane is produced from the methyl moiety of 1 molecule of acetate. The rate of [2-^{14}C]acetate consumption and methane production was monitored as described above. The theoretical amount of methane (M) produced from acetate (in micromoles per hour) was calculated by the relationship: M = acetate turnover (micromoles/milliliter/hour) × liquid volume in the vial (milliliters). The acetate contribution to methane (A [percentage]) was calculated by the relationship: $A = (M/\text{total methane production}) \times 100$.

RESULTS

Chemostat biomethanation performance. To assess the whey-processing chemostat performance, soluble metabolite concentrations, lactose conversion to methane and carbon dioxide, and electron and carbon recoveries were determined. The levels of metabolites in samples removed from the chemostat during steady-state conditions at a 100-h dilution rate were as follows (micromolar): acetate, 660 ± 300; propionate, 388 ± 55; lactate, 272 ± 17; butyrate, 100 ±

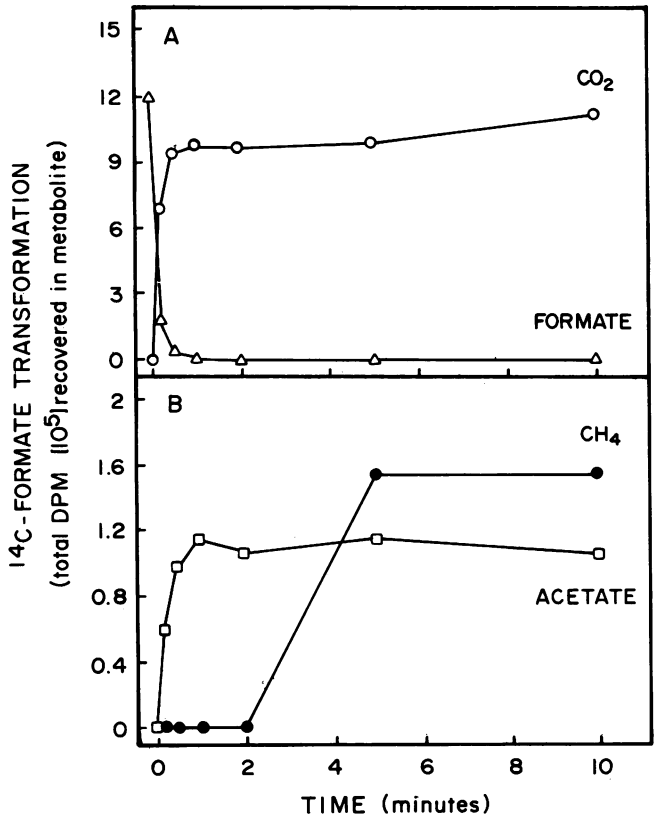

FIG. 5. Fermentation time course for [^{14}C]formate degradation by a whey-processing chemostat sample. The conditions were those described in the legend to Fig. 2.

70; ethanol, 16 ± 3; and hydrogen, 2.5 ± 0.08. Lactose and formate were below the detection limits of the enzymatic assay (14 μM). All metabolites were present at very low levels, with acetate and propionate predominating. The addition of exogenous lactose enhanced growth and methane production.

Table 1 presents a carbon and electron balance for lactose biomethanation by the whey-processing chemostat. The daily input of lactose was 1.42 mmol, and three major end products accounted for more than 98% of the lactose consumed. These were (in mmol per day): CH_4, 6.61; CO_2, 6.19; and cellular carbon, 1.64. The carbon recovery was 87%, and the electron recovery was 90%. The mass balance for 100 mM lactose was: 465 CH_4 + 436 CO_2 + 115 cellular carbon + 2.9 acetate + 1.1 lactate + 1.7 propionate + 0.42 butyrate + 0.07 ethanol + 0.011 H_2.

Lactose transformation route. ^{14}C tracer experiments were initiated to demonstrate the route of intermediary metabolite formation during lactose conversion to methane and carbon dioxide under steady-state conditions with a 100-h retention time. Short-term time courses with chemostat subsamples were performed so as to closely approximate the pool size and steady-state conditions.

Figure 2 shows the time course of ^{14}C-labeled intermediary metabolite formation from lactose to methane by continuous culture sample. Lactose was rapidly consumed during the first 3 min of incubation, and acetate, lactate, ethanol, and formate were the major labeled intermediary metabolites formed. Other products (i.e., valerate, propionate) were detected in experiments at background levels. Acetate concentration increased during the first 7 min and then remained constantly high during the time course. The formation of lactate was very rapid, and it was not possible to demonstrate the formation kinetics of this intermediary metabolite because after 1 min the level of lactate decreased steadily. The maximum concentration of formate was achieved after 5

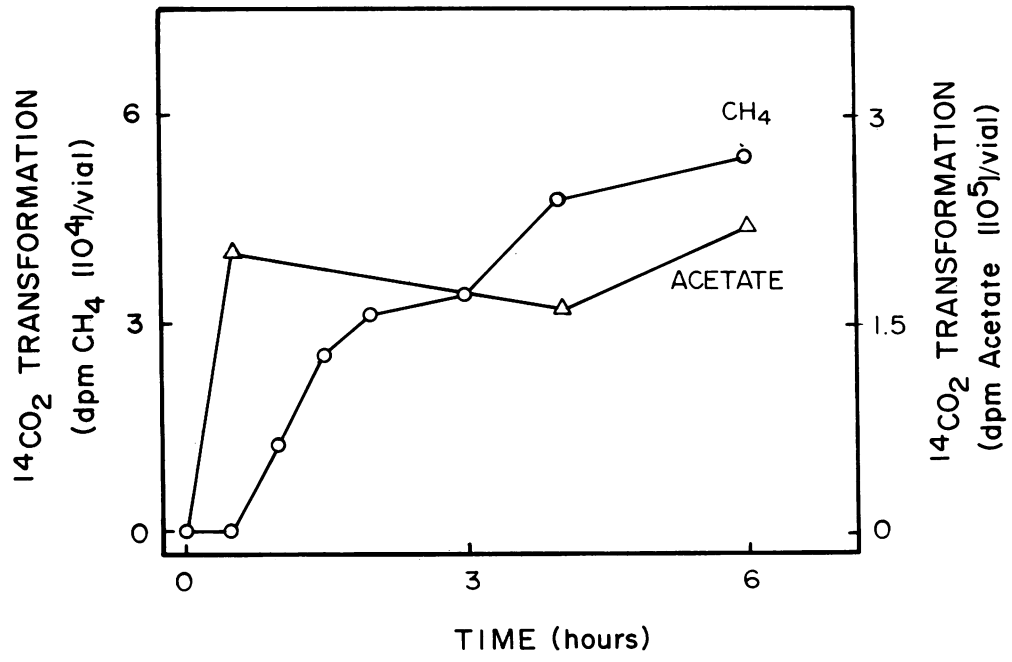

FIG. 6. Fermentation time course for $^{14}CO_2$ transformation by a whey-processing chemostat sample. The conditions were those described in the legend to Fig. 2.

min and then decreased below detection limits within 20 min. The maximum concentration of ethanol was achieved after 8 min and slowly decreased during the experiment. $^{14}CO_2$ production rapidly increased for 10 min and then reached a plateau, whereas methane formation slowly increased after 10 min. After 5 min of incubation, acetate accounted for more than 70% (in disintegrations per minute per vial) of the labeled metabolites formed from lactose.

Figure 3 shows the degradation time course of [U-^{14}C]lactate by a sample from the chemostat. Lactate was rapidly consumed during the first 3 min and was no longer detectable. Labeled acetate and, notably, ethanol were rapidly produced and reached a plateau after 3 min. Gas production from [^{14}C]lactate resembled that of [^{14}C]lactose.

Figure 4 shows the time-course degradation of [1-^{14}C]ethanol by a sample from the whey-processing chemostat. Ethanol was totally consumed during the first 8 min of incubation and formed acetate, CO_2, and CH_4. The formation of acetate appeared to be directly linked to ethanol disappearance. The rate of methane production was more rapid from ethanol than from lactate.

Figure 5 shows the time course of end product appearance from [^{14}C]formate by a sample from the chemostat. More than 95% of the formate was removed from the medium in 1 min. The major labeled metabolite produced from formate was CO_2, which accounted for more than 90% (in disintegrations per minute per vial) of the end products formed after 4 min. The production of $^{14}CO_2$ was coupled with the utilization of [^{14}C]formate. [^{14}C]acetate also reached its maximum concentration after 1 min, and only traces of methane were formed at 10 min, suggesting that formate was not a significant direct methane precursor.

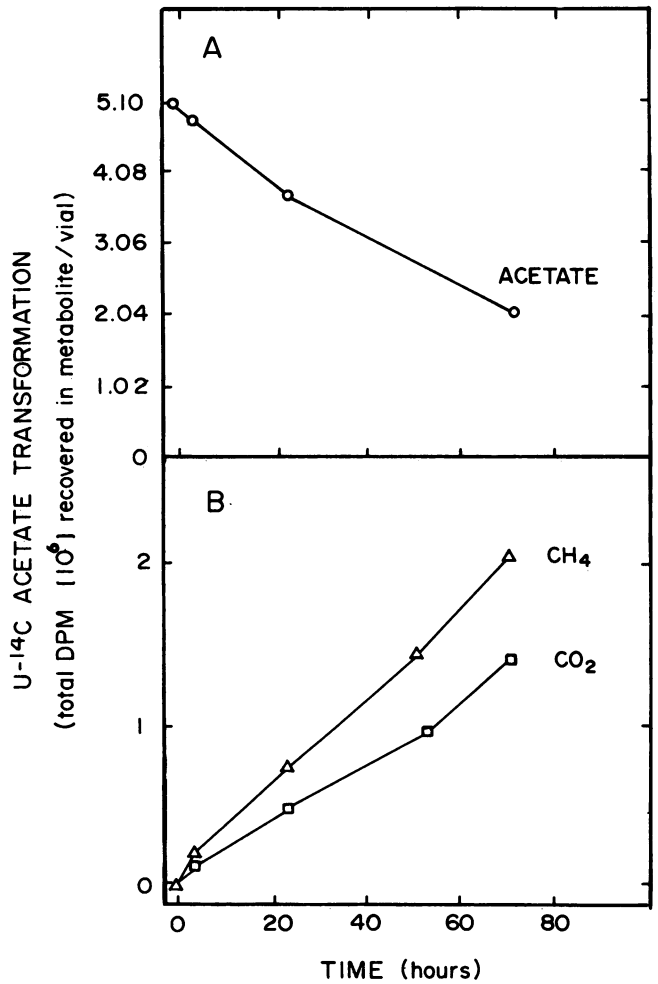

FIG. 7. Fermentation time course for [U-^{14}C]acetate degradation by a whey-processing chemostat sample. The conditions were those described in the legend to Fig. 2.

TABLE 2. Major carbon metabolite transformation kinetic parameters in a sample obtained from a whey-processing chemostat operated under substrate-limited conditions[a]

Metabolite	Substrate (μM)	Apparent kinetic parameters[b]	
		Turnover rate (μmol/h/ml)	Turnover rate constant (h^{-1})
Lactose	ND[c]		0.45
Lactate	120	1.47 ± 0.26	13.3 ± 2.16
Ethanol	12	0.24 ± 0.016	20.13 ± 1.31
Acetate	4,850	1.350 ± 0.15	0.28 ± 0.03

[a] The chemostat was operated at a 50-h retention time and was fed with PBB medium containing 10 g of dry whey per liter. The values are the average of two independent determinations. The correlation coefficients were greater than or equal to −0.98.

[b] The method assumes that the added tracers were in equilibrium with metabolic pools and approximates steady-state conditions. The values may underestimate the in situ turnover.

[c] ND, Not detectable.

Figure 6 shows the utilization of $^{14}CO_2$ by a sample from the whey-processing chemostat. The amount of $^{14}CO_2$ produced from dissolved carbonate rapidly increased in the headphase during the first 30 min. Labeled acetate was detected as the major soluble metabolite, but trace levels of propionate were also detected. The formation kinetics of labeled acetate were very rapid and reached a plateau after 30 min, which indicated a rapid exchange. The formation of labeled methane showed a constant increase during the entire incubation.

Figure 7 shows the utilization of [1,2-^{14}C]acetate by a sample from the chemostat. Acetate was slowly utilized, and nearly equal amounts of labeled CH_4 and CO_2 were produced as end products.

Carbon degradation rates. The rates of carbon degradation and the flux of carbon during lactose biomethanation were calculated by determining the turnover rates of lactose, lactate, ethanol, and acetate. The experiments were initiated in the same chemostat but were operated at 50-h retention time. No major differences were noticed in metabolite levels or in the lactose biomethanation parameters with a 50-h versus a 100-h retention time. Table 2 shows the turnover rates for the major intermediary metabolites. The input of lactose was 0.45 μmol/h per ml and was equal to its turnover. The turnover rates were (in micromoles per hour per milliliter): 1.47 for lactate, 0.24 for ethanol, and 1.350 for acetate. The amount of lactose directly transformed into lactate was 82%, and 81% of the methane was formed from acetate. It should be noted that these values represent the apparent kinetic parameters from carbon degradation under substrate-limited conditions. The real capacity for rates of carbon degradations is much higher for all metabolites under conditions that are not substrate limited (unpublished results).

DISCUSSION

The results presented here show that lactose transformation to methane and carbon dioxide in a steady-state whey-processing chemostat occurred as a result of the metabolic

integration of three simultaneous but distinct phases. First, in the hydrolytic phase, multiple fermentation products were formed from lactose: lactate, ethanol, acetate, formate, and CO_2. A second, acetogenic, phase converted these intermediary metabolites into acetate in H_2-CO_2. In the last phase, methanogenesis occurred from acetate and H_2-CO_2. In this ecosystem, lactate was the major intermediary metabolite of lactose fermentation, and acetate was the major immediate methane precursor. The contribution of acetate (81%) was greater than in values reported in several other ecosystems (3, 10, 15, 20, 25).

The analysis of lactose concentration in the whey-processing chemostat revealed that the substrate concentration was below the detection limits. The concentration of acetate and propionate was in the lower range of values reported for sewage sludge and whey digestors (23; Yang et al., Meet. Am. Soc. Agric. Eng., 1982). Lactate, ethanol, and butyrate were at very low concentrations. These low concentrations of metabolites show that carbon was effectively degraded to methane in this whey-processing chemostat in which the resident bacterial population was operating under substrate-limited conditions. The carbon balance of lactose biomethanation showed that the major products were methane (45%), carbon dioxide (42%), and cells (11%). The deviation from Buswell's theoretical equation (4), which predicts that 6 moles of CH_4 and 6 moles of CO_2 will be produced from lactose, may be explained by the high cell synthesis efficiency and low levels of other metabolites.

The biomethanation performance of the whey-processing chemostat is somewhat atypical when one considers the retention time. Most waste treatment digestors operate with retention times much longer (29 days) than 100 h, presumably because of uncoupling the rapid acidogenic phase from the slower methanogenic phase (21). In the present study, the lactose concentrations used were lower than those found in whey, and the practical significance of the results can be questioned. However, we will report later that the microbial population of this chemostat system can maintain effective biomethanation of raw whey with normal lactose concentrations at a 24-h dilution rate when the physiological requirements of the bacteria are satisfied (manuscript in preparation).

ACKNOWLEDGMENTS

This research was supported by grants from the Institute Pasteur, Societe Lyonnaise des Eaux, and the Michigan Biotechnology Institute.

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