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Sulfate Reduction and Anaerobic Glycerol Degradation by a Mixed Microbial Culture

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Abstract. Anaerobic glycerol degradation by a mixed microbial culture from a fermenter fed with industrial alcohol distillation waste water, was investigated in the absence or presence of sulfate, at 37°C and at a constant pH of 7.2. In the absence of sulfate, glycerol utilization was found to be characterized by the transient formation of 1,3-propanediol prior to propionate and acetate accumulation. In the presence of sulfate, 1,3-propanediol production was minor, and the carbon balance reflected a considerable accumulation of intermediate(s). A study of the role of sulfate reduction and methanogenesis on anaerobic 1,3-propanediol degradation showed that consumption of this substrate by the mixed microbial culture required a terminal electron acceptor. The number of fermentative and sulfate-reducing bacteria with glycerol or 1,3-propanediol as carbon and energy source revealed that sulfate-reducing bacteria outcompete fermentative bacteria for these substrates. The possible ecological role of sulfate-reducing bacteria in the metabolism of these reduced substrates is discussed.

The classical *Desulfovibrio* species utilize a rather limited range of electron donors for sulfate reduction. Lactate, pyruvate, ethanol, malate, formate, and hydrogen are the common energy substrates for these bacteria [27]. Recently, new types of *Desulfovibrio* species were isolated that utilize fructose [6, 26], sucrose [16], and amino acids [35].

It has also been recently reported that glycerol can be used as energy and carbon source by some *Desulfovibrio* species in pure culture [10, 17, 24–26, 28, 30, 31]. Little is known, however, about the utilization of glycerol by sulfate-reducing bacteria [31]. Glycerol is a major component of waste water from bioethanol production plants (distillery stillage), which also contain sulfate [2, 3]; and anaerobic digestion is one of the main purification processes used on these wastes [2, 3].

The purpose of the present study was to examine whether sulfate-reducing bacteria play a role in anaerobic glycerol and 1,3-propanediol digestion, and to quantify the populations involved in this process.

Materials and Methods

Sources of microorganisms. The microbial mixed culture came from a continuous fermenter (20-liter Biolafitte reactor; agitation rate, 50 rpm, temperature, 37°C; sequential feeding, 1 liter every 3 days with a mean organic load of 0.4 g COD $L^{-1} d^{-1}$ and hydrolic residence time of 60 days) fed with waste water from an industrial distillery (INRA-Narbonne, France). The main substrates present in the waste were glycerol and lactic acid. Concentrations of sulfate varied between 6 and 12 m*M*. The inoculum was from an anaerobic lagoon of distillery waste waters [2, 3].

Media. The basal sulfate-free salt solution mineral-medium used for batch culture experiments had the following composition (g/L): K_2HPO_4, 0.4; KH_2PO_4, 0.4; NH_4Cl, 0.9; NaCl, 0.9; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; cysteine-HCl · H_2O (as a reducing agent), 0.4; resazurin, 0.001. A basal bicarbonate buffered and sulfide-reduced medium with vitamins and trace elements was composed and prepared as described by Widdel and Pfennig [39] and modified by Widdel [37]; it was supplemented with 0.01% yeast extract (Difco) for fermentative and sulfate-reducing bacterial counts.

Growth conditions. Batch culture experiments were carried out at 37°C either in 1-L magnetically stirred reactors with pH control or in 60-ml serum bottles closed with butyl-rubber stoppers, incubated in a gyratory shaker, without pH control. Prior to the batch culture experiments, the inoculum was anaerobically diluted to 50% (vol/vol) with the sterilized sulfate-free salt solution under O_2-free N_2, with a Hungate technique [15] as modified for the use of syringes [20]. The diluted samples were incubated under 100% N_2, at 37°C, for 3 or 4 days to stimulate bacterial growth and to ensure that the sulfate derived from the samples was completely consumed. Samples were then distributed among reactors and

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bottles under anaerobic conditions. Organic substrates, sulfate, and chloroform (an inhibitor of methanogenesis) were added from separately sterilized stock solutions.

Bacterial counts. Fermentative and sulfate-reducing bacteria were counted by the most probable number (MPN) method with the following substrates: glycerol (20 m*M*) and 1,3-propanediol (20 m*M*). Sulfate-reducing bacteria were counted in the presence of sulfate (20 m*M*). Total flora was estimated by the acridine orange epifluorescence method [14]. The MPN tubes were incubated at 37°C for 4 weeks; growth of fermentative and sulfate-reducing bacteria was determined from optic density measurements at 580 nm in Bausch and Lomb Spectronic 70 spectrophotometer and sulfide production respectively. Metabolites produced from the various substrates were also determined.

Analytical methods. Sulfide production could not be demonstrated under these conditions by spectrophotometric methods because of the strong color of the waste used in the experiments. The sulfate reduction activity was, therefore, estimated from sulfate depletion in the culture medium. Sulfate was analyzed by the HPLC technique under the following conditions: pump, Shimadzu LC 6A; eluant, phthalic acid (3 m*M*) neutralized with Na-tetraborate at pH 4.9; flow rate, 2 ml min^{-1}; injection loop, 100 μl; column, Vydac anion exchange phase 302 IC (25 cm long); room temperature; conductivity detection was performed with a Shimadzu model CDD 6A detector; recorder integrator, Shimadzu chromatopak CR 3A; external standard. Sulfide was determined spectrophotometrically in the form of colloidal CuS, as described by Cord-Ruwisch [5]. Glycerol was determined by the enzymatic method developed by Batlle and Collon [1]. 1,3-propanediol was measured by means of FID gas chromatography (THEED 10% column on carbopack [SUPELCO, Inc.] 1 m long, 2 mm inner diameter; column temperature, 125°C; carrier gas, nitrogen; internal standard, ethyleneglycol). Volatile fatty acids were analyzed by means of FID gas chromatography (Glass column, 2 m long; 4 mm inner diameter; filled with Chromosorb (SUPELCO, Inc.), coated with 10% SP-1200 and 1% H_3PO_4; carrier gas, nitrogen; column temperature, 105°C; internal standard, ethyl 2-butyric acid. Alcohol and nonvolatile fatty acids were analyzed by HPLC measurements (column, Aminex HPX-87H (BIORAD, Richmond); detector, differential refractometer (Knauer); external standard). Methane and hydrogen were measured with TCD gas chromatography as described by Qatibi and Bories [29]. Total organic carbon (TOC) was measured on a Dohrmann DC 80 analyzer [18].

Results

Anaerobic glycerol degradation. In the absence of sulfate, with pH control at 7.2, glycerol was degraded with transient 1,3-propanediol formation before propionate and acetate production occurred; the 1,3-propanediol accumulated was slowly degraded into propionate and acetate (Fig. 1a). The kinetics of the total organic carbon measured and calculated revealed the presence of unknown intermediate(s) (Fig. 1b). In the presence of sulfate (Fig. 2a), with pH maintained at 7.2, glycerol was degraded and a parallel sulfate depletion occurred, coinciding with a transient formation of 1,3-propanediol and other intermediate(s), before propionate (then converted into acetate by sulfate reduction) and acetate production took place. A little 1,3-propanediol was produced, and the carbon balance (Fig. 2b) showed that considerable accumulation of the intermediate(s) had occurred.

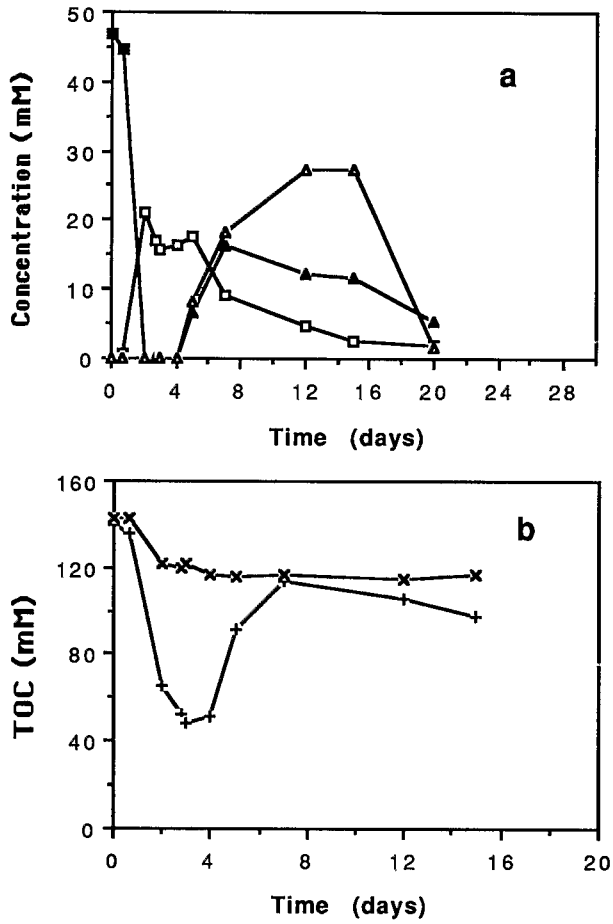

Fig. 1. Time course of anaerobic glycerol degradation by (a) the mixed microbial culture and (b) total organic carbon (TOC) calculated and measured. The experiment was performed in the absence of sulfate at constant temperature and pH (37°C and 7.2 respectively). Symbols: ■, glycerol; □, 1,3-propanediol; ▲, propionate; △, acetate; ×, TOC measured; +, TOC calculated.

Effects of sulfate reduction and methanogenesis on anaerobic 1,3-propanediol degradation. To determine the relationship between sulfate reduction and methanogenesis on anaerobic 1,3-propanediol degradation, experiments were carried out in which chloroform was added to inhibit methanogenesis (final concentration, 0.003% vol/vol) in the absence or presence of sulfate. As shown in Table 1, when

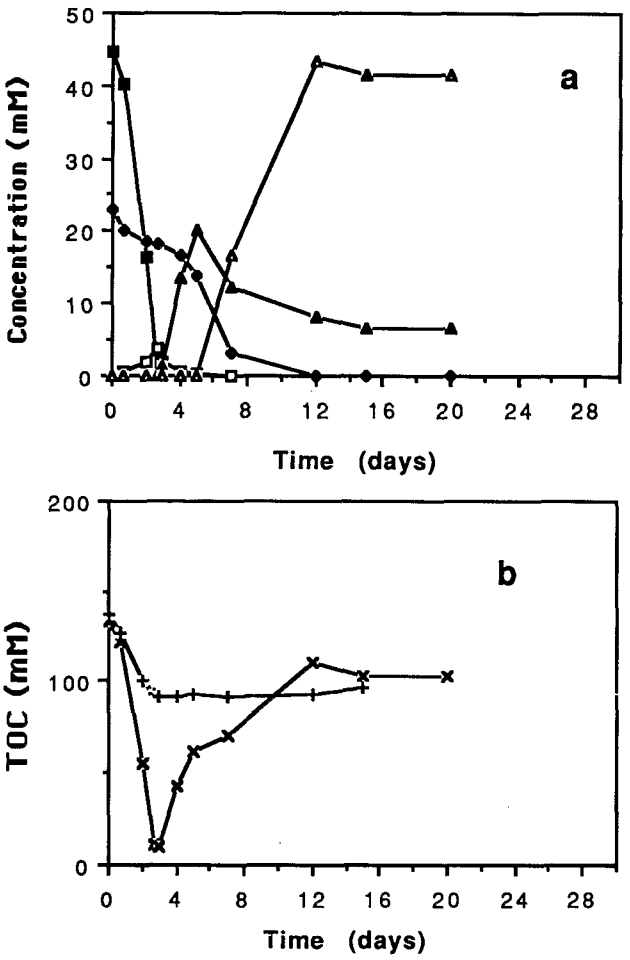

Fig. 2. Time course of anaerobic glycerol degradation by (a) the mixed microbial culture and (b) total organic carbon (TOC) calculated and measured. The experiment was performed in the presence of sulfate at constant temperature and pH (37°C and 7.2 respectively). Symbols: ■, glycerol; □, 1,3-propanediol; ▲, propionate; △, acetate; ◆, sulfate; ×, TOC measured; +, TOC calculated.

chloroform was added to the waste with or without sulfate, methane production from 1,3-propanediol as carbon and energy source was entirely inhibited. In the absence of sulfate, and in the presence of chloroform, hydrogen accumulated; it was consumed when sulfate was added, and the amount of 1,3-propanediol degraded was higher in the presence than in the absence of sulfate. This suggests that 1,3-propanediol degradation required either a terminal electron acceptor such as sulfate or syntrophic cooperation with methanogenic hydrogenotrophic bacteria.

Bacterial counts. Table 2 shows results from bacterial counts. In total, approximately 10^{10} bacterial cells were counted per ml by the epifluorescence technique with acridine orange. In low-sulfate glycerol medium, 2.5×10^9 fermentative bacteria were estimated per ml by MPN counts. In the last positive tubes, propionate, acetate, methane, and presumably carbon dioxide were produced. *Propionobacterium* sp., *Desulfovibrio* sp., and *Methanibacterium* sp. were observed by microscopic examination. In high-sulfate glycerol medium, 1.4×10^9 sulfate-reducing bacteria were counted per ml; acetate, sulfide, and presumably carbon dioxide accumulated. Species of *Desulfovibrio* were identified as the main glycerol degraders, from their morphology and fermentation products; 4.0×10^7 fermentative bacteria were counted per ml with 1,3-propanediol as sole substrate in the absence of sulfate. Putative species of *Pelobacter* and *Desulfovibrio* were observed in the last positive tubes; propionate, acetate, methane, and persumably carbon dioxide were produced, whereas in the presence of sulfate, 2.5×10^7 sulfate-reducing bacteria were estimated per ml. Species of *Desulfovibrio* were identified as the main 1,3-propanediol degraders in the last positive tubes with accumulation of acetate, sulfide, and presumably carbon dioxide.

Discussion

Anaerobic degradation of glycerol requires the reduction of electron acceptors such as fumarate, as occurs in *Escherichia coli* [32] or *Streptococcus faecalis* [13] or CO_2 in the case of homoacetogenic bacteria [8, 9]. The production of 1,3-propanediol as a reduced end product from glycerol has been observed in *Clostridium pasteurianum* [23], *Clostridium butylicum* [12], *Klebsiella pneumoniae* [11], *Lactobacillus brevis*, and *Lactobacillus buchneri* [33], *Citrobacter freundii* [22], and *Klebsiella aerogenes* [36]. In this study, it was demonstrated that in the absence of sulfate, anaerobic glycerol degradation by the mixed microbial culture studied was characterized by the transient formation of 1,3-propanediol prior to propionate and acetate accumulation. No lactate [19], ethanol and butyrate [12], acrolein [34], succinate, or pyruvate were produced. The presence of sulfate as a terminal electron acceptor seems to diminish the production of 1,3-propanediol by the mixed microbial culture. Thus, in the presence of sulfate, the problem of excess electron release during glycerol degradation is solved. These results suggest that sulfate-reducing bacteria may be involved in glycerol degradation with sulfate as terminal electron acceptor. The results of our enu-

Table 1. Effects of sulfate reduction and methanogenesis on anaerobic 1,3-propanediol degradation by the mixed microbial culture. Incubation at 37°C, for 4 weeks

Substrates	Initial concentration (m*M*)				Final concentration (m*M*)					
	1,3-OH[a]	C3	C2	sul	1,3-OH	C3	C2	sul	CH_4	H_2
1,3-OH	17.7	0	0	0	11	0.3	0	0	6.4	0
1,3-OH + sul	20.6	0	0	16.3	10.3	0.1	0	11.6	9.2	0
1,3-OH + chl	18.4	0	0	0	17.4	0.3	0.7	0	0	0.9
1,3-OH + sul + chl	18.6	0	0	15.9	9.2	0.1	0.3	6.7	0	0

[a] Symbols: 1,3-OH, 1,3-propanediol; C3, propionate; C2, acetate; sul, sulfate; chl, chloroform.

Table 2. MPN bacterial counts of fermentative and sulfate-reducing bacteria involved in anaerobic glycerol and 1,3-propanediol degradation by a mixed microbial culture.

Substrates	Bacterial counts (viable cells/ml)	Products of fermentation
− sulfate		
Glycerol	2.5×10^9	propionate + acetate + methane
1,3-propanediol	4.0×10^7	propionate + acetate + methane
+ sulfate		
Glycerol	1.4×10^9	sulfide + acetate[a]
1,3-propanediol	2.5×10^7	sulfide + acetate[a]
Total flora[b]	10^{10}	nd

[a] Traces of propionate were also detected.
[b] Estimated by the acridine epifluorescence method [15].

merations are consistent with this hypothesis. Sulfate-dependent glycerol oxidation was only recently unequivocally proved [10, 17, 24–26, 28, 30, 31, 35]. Furthermore, the values obtained in the absence of sulfate (53% carbon recovery when glycerol has disappeared, versus 91% at the end) and in the presence of sulfate (12.5% carbon recovery when glycerol has disappeared, versus 100% approximately at the end) are quite similar to those reported on pure culture of *Desulfovibrio carbinolicus* growing on glycerol with or without sulfate [24]; they strongly suggest the possibility that the intermediate produced during glycerol degradation by mixed microbial culture was 3-hydroxypropionate, although it is difficult to compare the results obtained because the media used were different, and the behavior of bacteria in pure culture differs greatly from that in mixed cultures.

Anaerobic oxidation of 1,3-propanediol has been reported with *D. carbinolicus* in the presence of sulfate [25] and with a strain of *Pelobacter carbinolicus* that oxidizes 1,3-propanediol into acetate only in the presence of a methanogenic hydrogenotrophic bacterium [7]. Our finding that 1,3-propanediol degradation did not occur when methanogenesis was inhibited in the absence of sulfate (Table 1) suggests that 1,3-propanediol degradation requires a terminal electron acceptor such as sulfate or a methanogenic hydrogenotrophic bacterium.

It is interesting to note that in low-sulfate medium tubes from enumerations with glycerol and 1,3-propanediol, *Desulfovibrio* species were observed by microscopic examination. It has, in fact, been demonstrated that *Desulfovibrio* species utilize glycerol and diols such as 1,2- and 1,3-propanediol by interspecies hydrogen transfer [28, 30, 31]. Our results on glycerol and 1,3-propanediol degradation by the mixed microbial culture agree with the concept that anaerobic degradation of reduced products of classical fermentations such as alcohols or fatty acids usually requires the reduction of electron acceptors such as sulfate [38], or a syntrophic association of fermenting and methanogenic bacteria, for example [4, 21]. Our results also show that glycerol and 1,3-propanediol (two substrates recently described as potential donors of reducing equivalents

for sulfate reduction) may be used by sulfate-reducing bacteria in the methanogenic ecosystem studied, possibly thanks to the presence of glycerol and sulfate in the original fermenter.

The isolation and characterization of the main sulfate-reducing bacteria using glycerol and 1,3-propanediol as energy and carbon sources from the positive tubes of enumerations will be described elsewhere [28].

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