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Glycerol and propanediols degradation by *Desulfovibrio alcoholovorans* in pure culture in the presence of sulfate, or in syntrophic association with *Methanospirillum hungatei*

A.I. Qatibi, J.L. Cayol and J.L. Garcia

Laboratoire de Microbiologie, ORSTOM, Université de Provence, Marseille, France

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1. SUMMARY

In a mineral medium containing sulfate as terminal electron acceptor, the sulfate-reducing bacterium *Desulfovibrio alcoholovorans* oxidized stoichiometrically 1 mol glycerol to 1 mol acetate and 1 mol 1,3-propanediol to 1 mol acetate with the concomitant reduction of 0.75 and 1 mol sulfate, respectively; 1 mol 1,2-propanediol was degraded to 0.8 mol acetate and 0.1 mol propionate, with the reduction of approximately 1 mol sulfate. The maximum specific growth rates (μ_{max} in h^{-1}) were 0.22, 0.086 and 0.09 with glycerol, 1,3-propanediol and 1,2-propanediol, respectively. The growth yields were 12.7 g, 11.1 g and 7.2 g dry weight/mol 1,3-propanediol, glycerol and 1,2-propanediol degraded, respectively. The growth yields and maximum specific growth rates of the H_2-transferring associations were also calculated. In the absence of sulfate, all these reduced substrates were degraded to acids and methane when *D. alcoholovorans* was cocultured with *Methanospirillum hungatei*. Changes in the metabolic pathway were observed in the degradation of 1,2- and 1,3-propanediol. The metabolic efficiency of *D. alcoholovorans* to degrade glycerol, 1.2- and 1,3-propanediol is discussed.

2. INTRODUCTION

Among the sulfate-reducing bacteria, the 'classical' species of the genus *Desulfovibrio* are known to grow on a limited range of oxidizable substrates including hydrogen, ethanol, lactate, formate, malate, fumarate and succinate [1]. Few data have been published on the utilization by this bacterial group of reduced products such as

Correspondence to: A.I. Qatibi. Present address: Department of Microbiology, University of Groningen, NL-9751 NN Haren, The Netherlands.

glycerol and diols such as 1,2-propanediol and 1,3-propanediol [2].

Glycerol poses a problem due to its redox state. Consequently, various fermentative bacteria can degrade glycerol only if terminal electron acceptors are present [3–6]. Several *Clostridium* species produced large amounts of 1,3-Propanediol as reduced product from glycerol [7]. Furthermore, although the major end product of glycerol metabolism was acetate in some homoacetogenic bacteria, small amounts of 1,3-propanediol were always formed as well [2,8]. 1,3-Propanediol is also a product that accumulates during anaerobic digestion of wastewater from bioethanol production plants [2,9,10]. 1,2-Propanediol is produced during glycerol degradation by some *Lactobacillus* species [11]. 1,2-Propanediol and 1,3-propanediol pose the same problem as glycerol and their degradation by a species of *Pelobacter* in association with a hydrogenotrophic methanogen has been described [12].

The following recently isolated species resemble the 'classical' *Desulfovibrio* species but have some deviating properties: *Desulfovibrio carbinolicus* [13,14] and *Desulfovibrio fructosovorans* [15] ferment glycerol in the absence of sulfate to 3-hydroxypropionate and 1,3-propanediol. In the presence of sulfate, *D. carbinolicus* oxidizes glycerol to 3-hydroxypropionate [14], whereas *D. fructosovorans* oxidizes this compound to acetate [15]. *D. carbinolicus* like *D. fructosovorans* is capable of oxidizing 1,3-propanediol to 3-hydroxypropionate, but unable to use 1,2-propanediol as energy source [2,14]. Furthermore, sulfate-dependent glycerol and diol oxidation have been recently found to occur in the anaerobic digestion of waste water from bioethanol production plants [2,10].

The purpose of the present study was to demonstrate the capacity of a new species, *Desulfovibrio alcoholovorans* [16], isolated from a fermenter fed with alcohol distillery waste water, to use these reduced substrates in pure culture or in syntrophic association with *Methanospirillum hungatei*. The maximum specific growth rates and growth yields were determined under both culture conditions with a view to tentatively assessing the metabolic efficiency.

3. MATERIALS AND METHODS

3.1. Source of microorganisms

Desulfovibrio alcoholovorans (DSM 5433) was isolated from a fermenter containing alcohol distillery waste water; its isolation and characterization have been described elsewhere [16]. *D. carbinolicus* (DSM 3852) was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, F.R.G.; *D. fructosovorans* (DSM 3604) and *Methanospirillum hungatei* were obtained from the collection at our laboratory.

3.2. Media and growth conditions

Hungate's anaerobic technique [17] as modified for the use of syringes [18] was used throughout the experiments. The composition of the basal medium with vitamins [19] and trace element solution SL 10 [20] has been described elsewhere [16]. Dithionite was omitted. After sterilization (110 °C for 40 min), the pH was adjusted to 7.2–7.3 with sterile solution of HCl (3M). Substrates were added from freshly prepared, anaerobically autoclaved solutions. *D. alcoholovorans, D. fructosovorans* and *D. carbinolicus* were grown in pure culture in the presence of sulfate at 35 °C in completely filled 100-ml serum bottles sealed with black rubber stoppers. In coculture experiments, *D. alcoholovorans* and *M. hungatei* were grown in the absence of sulfate in 500-ml serum bottles sealed with black rubber stoppers, with 200 ml medium under an atmosphere of N_2-CO_2 (80 : 20%). Monocultures of *D. alcoholovorans* and cocultures of *D. alcoholovorans* and *M. hungatei* grew satisfactorily in the defined medium, but in order to obtain large amounts of cells and to determine the growth yields, 0.01% yeast extract was added to the media. For coculture experiments, media were inoculated with 10% (v/v) each of *D. alcoholovorans* and *M. hungatei*. Adaptation of cocultures was achieved by repeated transfer onto appropriate substrates and controlled by checking the disappearance of substrate and determining methane production.

3.3. Cell material determinations

Growth with various concentrations of glycerol, 1,2-propanediol and 1,3-propanediol was de-

termined in screw-capped Hungate tubes by performing optical density measurements at 580 nm in a Bausch and Lomb Spectronic 70 spectrophotometer. Dry weight determinations were carried out using 2000-ml screw-capped bottle cultures with the various substrates, with and without a gas phase in the case of coculture and monoculture experiments, respectively. The cell material was harvested by centrifugation and washed twice with 50 mM potassium phosphate buffer, pH 7.0. The pellet was dried to constant weight at 80 °C.

3.4. Chemical determinations

Sulfide was determined spectrophotometrically as colloidal CuS [21]. Methane was measured by means of gas chromatography (Delsi series 30; injection; 200 °C; column: 3 m × 6 mm, stainless steel column, Porapack Q 80–100 mesh, 190 °C; carrier gas: N_2, flow rate: 30 ml/min; detection: flame ionization, 250 °C). Glycerol and diols were measured using HPLC (column: interaction cation-exchange ORH-801, 1 cm. o.d. × 30 cm ion-exclusion; column temperature: 65 °C; detection: differential refractometer, Knauer, Berlin; flow rate 0.8 ml/min). Propionate, acetate and 3-hydroxypropionate were determined by HPLC, with the same column (column temperature: 35 °C; detection: UV spectrophotometric detector at 210 nm SPD6A Module, Shimadzu Corporation, Kyoto, Japan).

4. RESULTS

4.1. Glycerol degradation

Fig. 1 shows the kinetics of glycerol dissimilation by *D. alcoholovorans* in the presence of sulfate (Fig. 1a) and in the presence of *M. hungatei* (Fig. 1b). Glycerol dissimilation by *D. alcoholovorans* in the presence of sulfate or in association with *M. hungatei* led to acetate plus sulfide or methane production, respectively, and presumably to that of carbon dioxide. The maximum specific growth rate of a pure culture of *D. alcoholovorans* in the presence of sulfate was 0.22 h^{-1}; the maximum cell yield was approximately 11 g dry weight/mol glycerol degraded (Table 1). In association with *M. hungatei* in the absence of sulfate, a maximum

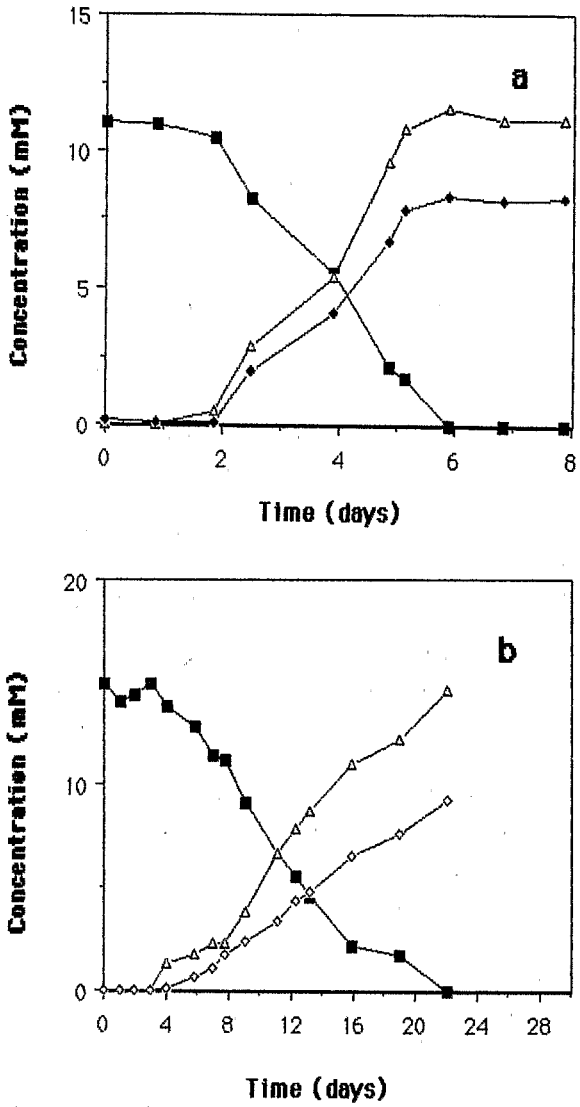

Fig. 1. Anaerobic degradation of glycerol by *D. alcoholovorans* in pure culture, in the presence of sulfate (a), or in coculture with *M. hungatei* (b). ■, glycerol; △, acetate; ◆, sulfide; ◇, methane.

specific growth rate of 0.047 h^{-1} and a maximum cell yield of 9.4 g dry weight/mol glycerol degraded were recorded (Table 1). From the results in Table 1, the stoichiometry in the presence of sulfate can be approximated by Eqn. 1 and by

Eqn. 2 in the coculture with *M. hungatei*:

$$CH_2OH\ CHOH\ CH_2OH + 0.75\ CO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75\ HS^- + 1.25\ H^+ + H_2O \quad (1)$$

$$CH_2OH\ CHOH\ CH_2OH \rightarrow CH_3COO^- + 0.25\ HCO_3^- + 0.75\ CH_4 + 1.25\ H^+ + 0.25\ H_2O \quad (2)$$

4.2. 1,2-Propanediol degradation

In the presence of sulfate, *D. alcoholovorans* degraded 1,2-propanediol to acetate, propionate and presumably CO_2 with production of sulfide (Fig. 2a). The acetate/propionate ratio was variable from one experiment to the other (see Fig. 2a and Table 1), probably because of the pH or redox potential of the media used. Apart from acetate and propionate, no other organic acid was detected. This strain did not produce any solvent such as propanol. The maximum specific growth rate was 0.09 h^{-1}. The maximum cell yield was approximately 7.2 g dry weight/mol 1,2-propanediol degraded (Table 1). As shown in Table 1, *D. alcoholovorans* degraded 1,2-propanediol as follows:

$$CH_2OH\ CHOH\ CH_3 + 1.025\ SO_4^{2-} \rightarrow 0.8\ CH_3COO^- + 0.1\ CH_3\ CH_2\ COO^- + 1.025\ HS^- + 0.975\ H^+ + H_2O + 1.1\ HCO_3^- \quad (3)$$

In the absence of sulfate, the hydrogen-consuming methanogenic bacterium *M. hungatei* served as an alternative acceptor for reducing equivalents released by *D. alcoholovorans* from 1,2-propanediol degradation (Fig. 2b). But only propionate was produced in addition to methane and presumably CO_2. The maximum specific growth rate of this coculture was 0.005 h^{-1}. The maximum cell yield was 2.8 g dry weight/mol 1,2-propanediol degraded (Table 1). The following theoretical stoichiometry can be proposed:

$$CH_2OH\ CHOH\ CH_3 + 0.25\ HCO_3^- \rightarrow CH_3\ CH_2\ COO^- + 0.25\ CH_4 + 0.75\ H^+ + 0.75\ H_2O \quad (4)$$

4.3. 1,3-Propanediol degradation

As expected, 1,3-propanediol degradation led to higher sulfide or methane production than glycerol degradation. At the beginning of incubation, in the presence of sulfate, 1,3-propanediol degradation led only to acetate, sulfide, and presumably CO_2 production (Fig. 3a). After about 4 mmol 1,3-propanediol per litre had been degraded, 3-hydroxypropionate was formed as a further end-product with an acetate/3-hydroxypropionate ratio of approximately 3.4. The maximum of 3-hydroxypropionate produced was approximately 1.2 mmol per litre (Fig. 3a). The maximum

Table 1

Yield determinations and stoichiometry [a] for growth of *D. alcoholovorans* on glycerol, 1,2-propanediol and 1,3-propanediol in the presence of sulfate (lines 1, 3 and 5) or in coculture with *M. hungatei* in the absence of sulfate (lines 2, 4 and 6)

Substrate	1	2	3	4	5	6	7	8	9
	Substrate degraded (mM)	OD 580	Cell material formed (mg/mol)	Cell yield (g/mol)	Cell formed expressed as acetate [b] (mM)	Acetate (mM)	Propionate (mM)	H_2S (S) or CH_4 (M) (mM)	e- recovery [c] (%)
Glycerol	10	0.26	110.6	11.1	2.3	7.9	0	6.5 (S)	96
Glycerol	8.8	0.21	82.7	9.4	1.7	7	0	5.30 (M)	91
1.2-propanediol	10	0.18	72	7.2	1.5	7.7	1.1	6.9 (S)	90
1.2-propanediol	10	0.055	27.5	2.8	0.6	0	10.5	1.5 (M)	102
1.3-propanediol	10	0.28	127.3	12.7	2.6	7.2	0	7.1 (S)	85
1.3-propanediol	10	0.24	102.6	10.3	2.1	7.6	0	7.6 (M)	86

[a] Bicarbonate present; [b] calculated from column 3 and the following equation:
$17\ CH_3COO^- + 11\ H_2O \rightarrow 8\ C_4H_7O_3 + 2HCO_3^- + 150H^-$ [41];
[c] calculated from columns 1,5, 6, 7 and 8.

specific growth rate of *D. alcoholovorans* on 1,3-propanediol in the presence of sulfate was about 0.086 h^{-1}. The maximum cell yield was 12.7 g dry weight/mol 1,3-propanediol degraded (Table 1). Eqn. 5 shows the stoichiometry of the degradation of 1,3-propanediol by a pure culture of *D. alcoholovorans* in the

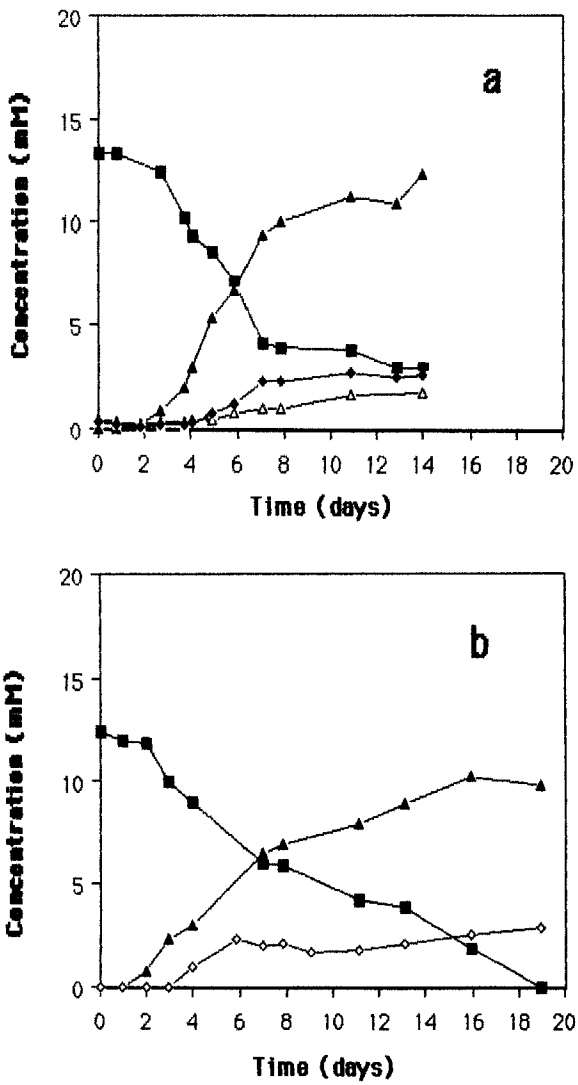

Fig. 2. Anaerobic degradation of 1,2-propanediol by *D. alcoholovorans* in pure culture, in the presence of sulfate (a), or in coculture with *M. hungatei* (b). ■, 1,2-propanediol; ▲, propionate; △, acetate; ◆, sulfide; ◇, methane.

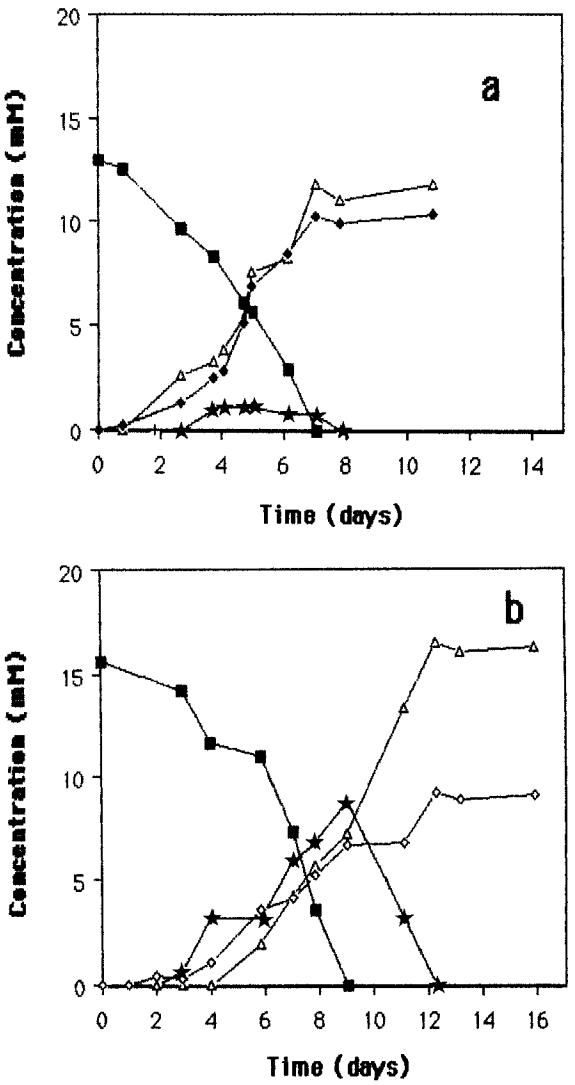

Fig. 3. Anaerobic degradation of 1,3-propanediol by *D. alcoholovorans* in pure culture, in the presence of sulfate (a), or in coculture with *M. hungatei*. (b) ■, 1,3-propanediol; ★, 3-hydroxypropionate; △, acetate; ◆, sulfide; ◇, methane.

presence of sulfate:

$$CH_2OH\ CH_2\ CH_2OH + SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + HS^- + H^+ + H_2O \quad (5)$$

Without sulfate and in coculture with *M. hungatei* 1,3-propanediol was first degraded to acetate, 3-hydroxypropionate, methane and pre-

Table 2

Products of 1,3-propanediol degradation by *Desulfovibrio* species in the presence of sulfate (15 mM)

Strains	1,3-OH degraded (mM)	Products (mM)		
		3-OHC3	C2	H2S
D. carbinolicus	10	9.8	0.0	4.4
D. fructosovorans	10	9.6	0.0	4.5
D. alcoholovorans	10	0.0	9.7	8.7

1,3-OH, 1,3-propanediol; 3-OHC3, 3-hydroxypropionate; C2, acetate.

sumably CO_2 with an acetate/3-hydroxypropionate ratio of approximately 1 (Fig. 3b). 3-Hydroxypropionate was then degraded to acetate and methane. The maximum specific growth rate of this coculture was 0.05 h^{-1}. The maximum cell yield was approximately 10 g dry weight/mol 1,3-propanediol degraded (Table 1). The stoichiometry of the degradation of 1,3-propanediol by the coculture may have been as follows:

$$CH_2OH\ CH_2\ CH_2OH \rightarrow CH_3COO^- + H^+ + CH_4 \quad (6)$$

On the one hand, 3-hydroxypropionate is only an intermediate compound in the degradation of 1,3-propanediol by *D. alcoholovorans* and its concentration depends on whether the terminal electron acceptor is sulfate (Fig. 3a) or protons in the case of syntrophic association between *D. alcoholovorans* and *M. hungatei* (Fig. 3b). On the other hand, 3-hydroxypropionate is the final product of the degradation of 1,3-propanediol by *D. fructosovorans* and *D. carbinolicus* (Table 2), even in the presence of excess sulfate. The degradation of 1,3-propanediol by *D. alcoholovorans* results in approximately twice as much sulfide as the degradation by *D. fructosovorans* and *D. carbinolicus* (Table 2).

5. DISCUSSION

Anaerobic degradation of glycerol often leads to the accumulation of 1,3-propanediol and propionate [2,22]. Sulfate-reducing bacteria able to oxidize 1,3-propanediol with or without sulfate have been isolated [2], such as *D. alcoholovorans* [16]. This bacterium also grew on 1,2-propanediol in monoculture and formed a mixture of acetate and propionate. If this bacterium is cocultured with *M. hungatei* on 1,2-propanediol, only propionate is produced; in this case, *M. hungatei* may have been unable to decrease the partial pressure of hydrogen sufficiently for proper interspecies hydrogen transfer and allow sufficiently strong activity of the enzymes of the acetate synthesis pathway. A similar phenomenon has been observed during glutamate fermentation by *Acidaminobacter hydrogenoformans* in pure culture or in coculture with *Methanobrevibacter arboriphilus* [23].

Dehydratation of 1,2-diols is carried out by fermentative bacteria such as *Clostridium glycolicum* [24], *Pelobacter venetianus* [25], *Pelobacter carbinolicus* [12,26], some species of *Lactobacillus* [11], *Klebsiella* [27], *Acetobacter* [28], *Propionibacterium* [29], and *Acetobacterium* [30]. Some of them possess a diol dehydratase which is involved in the metabolism of ethyleneglycol and 1,2-propanediol; it is probable that *D. alcoholovorans* also possess this enzyme. This can also explain the use of 1,2-propanediol by our strain. Propionate might be produced by a non-specific acetaldehyde dehydrogenase [27,31]; in this case, propionaldehyde would be an intermediate compound [32,33].

3-Hydroxypropionate seems to be an intermediate product of the degradation of 1,3-propane-diol. *D. alcoholovorans* might have a primary alcohol dehydrogenase and an aldehyde dehydrogenase oxidizing 1,3-diols as *P. carbinolicus* [12]. 3-Hydroxypropionate is converted to acetate, probably with malonylsemialdehyde as intermediate compound (see Doelle [3]). 3-Hydroxypropionate is the terminal product of the degradation of 1,3-propanediol by *D. carbinolicus* and *D. fructosovorans*, but can be degraded to acetate by *D. alcoholovorans*. The stoichiometry of 1,3-propanediol degradation to acetate by *D. alcoholovorans* with sulfate suggests the presence of the same enzymes and metabolic pathways as those known to exist in *Desulfovibrio* strain OttPd1 [35].

Our results show the requirement for a terminal electron acceptor for the oxidation of these reduced compounds; this acceptor may be either

sulfate [36] or a hydrogenotrophic methanogen [37,38]. This is in agreement with the results of Eichler and Schink [26] on the oxidation of primary aliphatic alcohols.

Generally, whatever substrate used, the speed of degradation will depend on the nature of the terminal electron acceptor [39]. Moreover, the replacement of a high potential terminal electron acceptor (sulfate) by a low potential acceptor (proton) results in deviations of metabolic pathways; so, incomplete interspecies hydrogen transfer is indicated by partial reduction of intermediary products of the metabolism of 1,2- and 1,3-propanediol.

The highest growth yield of *D. alcoholovorans* in pure culture with sulfate was obtained on 1,3-propanediol (12.7 g/mol); an intermediary yield was observed on glycerol (11.1 g/mol), and the lowest yield was measured on 1,2-propanediol (7.2 g/mol).

The stoichiometry of the degradation of glycerol and the fact that glycerol is converted entirely to acetate by *D. alcoholovorans* suggest that our strain possesses the same enzymatic equipment and the same metabolic pathways (for oxidizing glycerol to acetate) than two marine strains of *Desulfovibrio* studied by Kremer and Hansen [40].

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