Production of 1,3-Propanediol from Glycerol by *Clostridium acetobutylicum* and Other *Clostridium* Species

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Glycerol was fermented with the production of 1,3-propanediol as the major fermentation product by four strains of *Clostridium acetobutylicum*, six of *C. butylicum*, two of *C. beijerinckii*, one of *C. kainantoi*, and three of *C. butyricum*. 1,3-Propanediol was identified by its retention times in gas chromatography and high-pressure liquid chromatography and by its mass spectrum. During growth of *C. butylicum* B593 in a chemostat culture at pH 6.5, 61% of the glycerol fermented was converted to 1,3-propanediol. When the pH was decreased to 4.9, growth and 1,3-propanediol production were substantially reduced.

Clostridium acetobutylicum and *C. butylicum* strains are able to metabolize a wide range of carbohydrates, including hexoses and pentoses (4), hemicellulosic materials (8), and cellulosic materials, under suitable conditions of growth (7). The fermentation products usually include acetic acid, butyric acid, ethanol, acetone, butanol, and acetoin (4). However, when cells were grown on rhamnose, they also synthesized 1,2-propanediol, propionic acid, and propanol (4).

It has been reported that a number of bacteria, including *C. pasteurianum* (10), *Klebsiella pneumoniae* (3), and *Lactobacillus* species (11), ferment glycerol with the production of many fermentation products, including 1,3-propanediol. The objective of this investigation was to determine whether solventogenic strains of *C. acetobutylicum*, *C. butylicum*, and other *Clostridium* species could also ferment glycerol to produce derivatives of possible importance to the chemical industry.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial cultures used were those described by Lee et al. (7). They were grown at 35°C in a basal chemically defined medium (7) with 0.2% (wt/vol) each of yeast extract and sodium bicarbonate and with oxygen-free carbon dioxide as the gas phase. However, in one set of experiments, yeast extract was replaced by cobalt chloride hexahydrate and cobalamin, which were added to give final concentrations of 0.01 g liter⁻¹ and 0.5 mg liter⁻¹, respectively. Cultures were inoculated as a 10% (vol/vol) inoculum into 4.5 ml of medium and incubated with shaking (125 cycles min⁻¹) to test for glycerol utilization. For growth curves, 300-ml volumes of medium in 500-ml round-bottomed flasks were inoculated with 5% (vol/vol) quantities of 18-h cultures grown in the same medium.

Chemostat culture experiments were conducted as described by Lee et al. (7). The medium used was that described above, with 0.2% each of yeast extract and sodium bicarbonate.

Analysis of fermentation products. Fermentation products were analyzed by gas chromatography (GC) and high-pressure liquid chromatography (HPLC) (4, 6). For GC, 0.2-ml samples of cell culture fluid were removed, and 25 µl each of water and the internal standard, 0.1 M isovaleric acid, was added. After being mixed, 1 µl of the solution was injected into a GC (Hewlett-Packard model 5721A equipped with a

flame ionization detector and connected to a Hewlett-Packard model 3390 recording integrator). The fatty acids and solvents were separated in a glass column (2 mm [inside diameter] by 2 m [length]) freshly packed with Chromosorb 101 (80/100 mesh). Nitrogen at 20 ml min⁻¹ was used as the carrier gas. The injector and detector temperatures were 200 and 250°C, respectively. The oven temperature was programmed from 150 to 185°C at the rate of 12°C min⁻¹. The ratios of the retention times of the standards to that of the internal standard, isovaleric acid, were as follows: ethanol, 0.16; acetone, 0.21; *n*-propanol, 0.28; acetic acid, 0.38; *n*-butanol, 0.42; propionic acid, 0.54; acetoin, 0.61; 1,2-propanediol, 0.77; butyric acid, 0.77; 1,3-propanediol, 1.2; valeric acid, 1.27; caproic acid, 2.02; and glycerol, 3.4. Acidification of samples with sulfuric acid led to destruction of the diol compounds during chromatography.

For HPLC analysis, cell culture fluid was treated with 5% (wt/vol) Amberlite IR-120 (H⁺ form; BDH, Poole, England), and 10 µl was injected into an Aminex HPX-87H column [Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada] at 35°C and a flow rate of filtered, degassed 0.01 M sulfuric acid of 0.4 ml min⁻¹. Sample components were detected with a model 6510 Erma refractive index detector (Erma Optical Works, Tokyo, Japan) linked to a model 730 Waters recording integrator (Waters Scientific Ltd., Mississauga, Ontario, Canada).

The identification of 1,3-propanediol was accomplished by using a GC-mass spectrophotometer (MS) data system. Electron impact mass spectra were recorded at 70 eV on a model 12000 Micromass Quadrupole mass spectrometer (VG Masslab, Altringham, Cheshire, England) interfaced with a Hewlett-Packard model 5790 GC equipped with a DB-5 fused silica capillary column (Chromatographic Specialties, Brockville, Ontario, Canada). The GC column temperature was initially 50°C and was increased at 10°C per min to 320°C. Samples for analysis were prepared by freeze-drying extracellular culture fluid from a chemostat culture of *C. butylicum* B593 grown in a medium containing 2% (wt/vol) glycerol. The freeze-dried material was suspended in a one-half volume of anhydrous ethanol and centrifuged to remove insoluble material. The only components in the ethanol extracts detected by the GC system used were butyric acid, 1,3-propanediol, and glycerol. An authentic standard of 1,3-propanediol was also analyzed. The identities of compounds analyzed by GC-MS were confirmed by reference to *Compilation of Mass Spectral Data* (1).

TABLE 1. Fermentation products of *C. butylicum* B593 and 33007 during batch culture growth in a chemically defined medium with 1% (wt/vol) glycerol as the source of carbon and energy^a

Product	Concn (mM) of product produced in:			
	B593		33007	
	Unamended medium	Medium + 1,2-propanediol ^b	Unamended medium	Medium + 1,2-propanediol ^b
Acetic acid	6.02	11.18	6.15	2.00
Propionic acid	3.04	0.30	4.24	8.66
Butyric acid	11.25	18.14	12.49	42.30
Ethanol	0.54	0.12	0.52	— ^c
Acetone	<0.05	0.18	<0.05	3.50
Propanol	<0.05	90.70	0.08	21.19
Butanol	<0.05	<0.05	<0.05	<0.05
1,3-Propanediol	67.05	15.14	77.80	2.12
1,2-Propanediol	<0.05	36.14	<0.05	97.24

^a Sodium bicarbonate was included at a concentration of 0.2% (wt/vol), and CO₂ was the gas phase. The cultures were analyzed after 96 h of growth. Neither acetoin nor isovaleric acid was detected in the culture fluid.

^b Added to give a final concentration of 1% (wt/vol).

^c —, Not determined.

Glycerol. Glycerol present in cell culture fluid was analyzed either directly by HPLC or enzymatically as described by Eggstein and Kuhlmann (2). It could not be analyzed by GC because of extensive thermal destruction during the relatively long chromatographic separation period used.

RESULTS

Fermentation of glycerol by *Clostridium* species. Twenty-one strains of *Clostridium* spp. were screened for growth on glycerol and for the synthesis of 1,3-propanediol. The medium used was a basal chemically defined medium with 1% glycerol, 0.2% yeast extract, 0.2% sodium bicarbonate, and CO₂ as the gas phase. Of the cultures tested for growth, 17 grew through four serial subcultures. These included *C. acetobutylicum* IFO 3853, IFO 3854, NRRL B527, and ATCC 824; *C. butylicum* NRRL B592, NRRL B593, ATCC 14823, NRC 33005, NRC 33006, and NRC 33007; *C. butyricum* IFO 3315, IFO 3858, and CBS 31; *C. beijerinckii* ATCC 11914 and ATCC 14949; and *C. kaimantoi* IFO 3353.

Cultures which did not grow in this medium included *C. acetobutylicum* ATCC 10132 and ATCC 4259 and *C. beijerinckii* ATCC 858 and ATCC 25752. The cell culture supernatant samples from stationary-phase cultures of the third serial subculture were analyzed for fermentation products. All had similar product profiles, and 1,3-propanediol and butyric acid being the major fermentation products. *C. butyricum* IFO 3858 produced more butanol from glycerol than did the other bacteria tested.

Identification of 1,3-propanediol. When *C. butylicum* B593 and *C. butylicum* 33007 were grown in a chemically defined medium with glycerol as the sole source of carbon and energy, the major fermentation product detected was 1,3-propanediol (Table 1). In GC with Chromosorb 101 and a temperature gradient, 1,3-propanediol had a retention time of 1.2 relative to the internal standard, isovalerate, whereas the corresponding value for 1,2-propanediol was 0.77. In HPLC with an ion-exchange column for the separation of organic acids, the elution times for glycerol and 1,3-

FIG. 1. HPLC separation of products from the fermentation of glycerol by *C. butylicum* B593. The number beside each peak is the elution time in minutes. The numbers in large print above some peaks indicate the component separated: 1, anions; 2, anions; 3, glycerol; 4, 1,3-propanediol; 5, butyric acid and acetone.

TABLE 2. Fermentation products from the fermentation of glycerol by *C. butylicum* B593 during steady-state growth in a chemostat culture^a

Medium glycerol concn (mM)	pH	Optical density at 675 nm	Glycerol used (mM)	Concn (mM) of:							% Conversion to diol	% Carbon recovery ^b
				Lactic acid	Acetic acid	Butyric acid	Ethanol	Acetone	Butanol	1,3-Propanediol		
208.6 (1.9%)	6.5	3.0	203.3	9.7	1.7	19.7	0.9	<0.1	0.3	124.7	61.3	80.1
417.2 (3.8%)	6.6	6.8	406.0	3.5	26.9	6.6	2.3	0.4	2.4	247.6	61.0	70.0
417.2 (3.8%)	5.6	2.6	182.7	<0.1	23.1	10.5	0.4	<0.1	0.3	97.6	53.4	69.8
417.2 (3.8%)	4.9	2.3	160.1	0.2	19.3	11.7	0.6	<0.1	0.3	67.9	42.4	60.8

^a The dilution rate was 0.1 h⁻¹.^b Exclusive of carbon dioxide.

propanediol were 21.05 and 27.6 min, respectively (Fig. 1), whereas that for 1,2-propanediol was 26.65 min (data not shown). An ethanol extract of freeze-dried culture fluid from a chemostat culture on 2% glycerol (Table 2) was analyzed by both GC and GC-MS. GC analysis on a Chromosorb 101 column revealed three peaks corresponding to butyrate, 1,3-propanediol, and glycerol. Analysis by GC-MS revealed four peaks at scan numbers 71, 82, 114, and 265 on the DB-5 separating column (Fig. 2A). The four peaks (Fig. 2A) yielded the following *m/e* spectral peaks with greater than 25% abundance, presented in decreasing order: scan 71 (60, 27, 29, 73, 45, and 42), scan 82 (Fig. 2B) (31, 57, 58, 29, and 27), scan 114 (31, 57, 58, 29, and 27), and scan 265 (29, 43, 61, 30, 44, and 31). Scan 71 was characteristic of butyric acid, scans 82 and 114 were characteristic of 1,3-propanediol, and scan 265 was characteristic of glycerol. In other GC-MS runs

with the same separating column and identical chromatographic conditions, a single peak for 1,3-propanediol was observed.

Production of 1,3-propanediol from glycerol. The production of 1,3-propanediol by *C. butylicum* B593 during growth in a batch culture is shown in Fig. 3. Growth was exponential until an optical density at 675 nm of 4.0 was reached at approximately 7 h after inoculation. Similarly, the 1,3-propanediol concentration increased in an exponential fashion, 1,3-propanediol appeared after growth had commenced, although the rate of increase was greater than that observed for growth, and the eventual decrease in synthesis preceded the deceleration in growth as the stationary phase was reached. Glycerol utilization and butyrate production continued during the stationary phase. The continued synthesis of butyrate appeared to be at the expense of acetate, since the acetate concentration decreased during the stationary phase.

Fermentation product analysis of culture fluid from *C. butylicum* B593 and 33007 grown in batch cultures is shown in Table 1. Cells grown on glycerol alone produced a large amount of 1,3-propanediol and relatively low levels of fatty acids, and the solvents acetone and *n*-butanol were each present at concentrations of less than 0.05 mM. A low concentration of propionic acid was detected, but no *n*-

FIG. 2. GC-MS analysis of the less volatile fermentation products from *C. butylicum* B593. A 2-ml sample of culture supernatant from a glycerol-limited chemostat culture (pH 6.5; dilution rate, 0.1 h⁻¹) was lyophilized. The residue was suspended in a one-half volume of ethanol. (A) GC separation of products. Scan number 71 is butyrate, scan numbers 82 and 114 are 1,3-propanediol, and scan number 265 is glycerol. (B) Spectra for peak scan number 82.

FIG. 3. Batch culture fermentation of glycerol by *C. butylicum* B593. The medium was a basal chemically defined medium with 2% glycerol as the primary carbon source and 0.2% each of yeast extract and sodium bicarbonate. The gas phase was oxygen-free carbon dioxide. Symbols: ▲, glycerol; □, 1,3-propanediol; ●, optical density at 675 nm; △, butyric acid; ○, acetic acid.

propanol was detected. Growth of cells in the presence of 1,2-propanediol modified the fermentation product profile. The concentration of 1,3-propanediol was reduced with increased concentrations of butyric acid and *n*-propanol.

Growth of strains B593 and 33007 was somewhat erratic in the basal chemically defined medium without added yeast extract. When cobalt chloride and cobalamin were included in the culture medium for B593, good growth was obtained in the absence of added yeast extract. The inclusion of 0.2% (wt/vol) sodium bicarbonate and the use of carbon dioxide as the gas phase allowed double the growth as compared with that in media without bicarbonate and with nitrogen as the gas phase. This result presumably was due to the enhanced buffering capacity of the medium.

Chemostat cultures on glycerol. To determine the influence of culture conditions on the production of 1,3-propanediol, we grew *C. butylicum* B593 in continuous cultures at various pH values and glycerol concentrations (Table 2). At pH values of 6.5 and 6.6, 97% of the glycerol was metabolized, with 61% of the metabolized glycerol being converted to 1,3-propanediol. This estimation is based on the assumption that neither the sodium bicarbonate nor the yeast extract in the medium was converted to 1,3-propanediol. When the pH was lowered from 6.6 to 5.6 and then to 4.9, growth was reduced, and the efficiency of conversion of glycerol to 1,3-propanediol was decreased.

DISCUSSION

It was demonstrated in this study that *C. butylicum* and other solventogenic *Clostridium* species grow with glycerol as the sole source of carbon and energy and synthesize 1,3-propanediol as a major fermentation product, accounting for up to 61% of the glycerol used. This metabolic capability is shared with *C. pasteurianum* (10), *K. pneumoniae* (3), and *Lactobacillus* species (11); however, the efficiencies of the transformations differ. *C. pasteurianum* converts glycerol to 1,3-propanediol with an efficiency of approximately 15%, although a comparison to results of the present study is complicated because the medium used is different. *Citrobacter freundii* (9) and *K. aerogenes* (12) have been reported to convert glycerol to 1,3-propanediol during growth with efficiencies of up to 60 and 40%, respectively. In contrast to these bacteria, *Lactobacillus* species are unable to utilize glycerol as a source of carbon and energy for growth, but they convert it to 1,3-propanediol with greater than 80% efficiency when glucose is present as a carbon source (11).

The pathway for the conversion of glycerol to 1,3-propanediol has been established for *Klebsiella* spp. It involves the dehydration of glycerol by glycerol dehydratase to form 3-hydroxypropionaldehyde, which is subsequently reduced to 1,3-propanediol by an NAD-linked glycol dehydrogenase (3). The glycerol fermentation is dependent on cobalt because the dehydratase is a vitamin B₁₂-dependent enzyme.

It was observed that the inclusion of 1,2-propanediol in the culture medium inhibited glycerol conversion to 1,3-propanediol in *C. butylicum* B593. Forage and Foster (3) reported that both the glycerol and 1,2-propanediol dehydratases are induced in *K. pneumoniae* growing on glycerol. In the case of *C. butylicum*, when both substrates were present, the dehydratase and dehydrogenase activities of the respective pathways were induced. In *Propionibacterium freudenreichii* (5) and *K. pneumoniae* (13), 1,2-propanediol is dehydrated to propionaldehyde and either is

reduced to *n*-propanol by an NAD-linked dehydrogenase or is converted by a multistep pathway to propionate (13). Presumably, a similar set of metabolic processes occurs in *Clostridium* species. 1,2-Propanediol included in the medium containing glycerol may have provided an alternate electron acceptor that competed for electrons generated during the oxidation of the substrate, thereby leading to a reduced concentration of 1,3-propanediol and the conversion of 1,2-propanediol to *n*-propanol.

The reduced growth of *C. butylicum* on glycerol in a chemostat culture when the pH was decreased from 6.6 to 5.6 and lower indicates that the enzymes involved in glycerol metabolism or their synthetic systems are pH sensitive. This is probably of particular importance in the pathway leading to 1,3-propanediol synthesis, since the relative yield of 1,3-propanediol was reduced to a greater extent than was growth. *n*-Butanol production often is induced when the culture pH drops below 5.5 (6). The lack of *n*-butanol production from glycerol at pH 4.9 presumably was due to the diversion of reducing power from butyrate reduction to the reduction of 3-hydroxypropionaldehyde.

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