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Fermentation of raw glycerol to 1,3-propanediol by new strains of *Clostridium butyricum*

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Industrial glycerol obtained through the transesterification process using rapeseed oil did not support growth of several strains of *Clostridium butyricum* obtained from bacterial culture collections. Ten new strains of *C. butyricum* were obtained from mud samples from a river, a stagnant pond, and a dry canal. These new isolates fermented the commercial glycerol and produced 1,3-propanediol as a major fermentation product with concomitant production of acetic and butyric acids. Four of the ten isolates were able to grow on industrial glycerol obtained from rapeseed oil. One strain, *C. butyricum* E5, was very resistant to high levels of glycerol and 1,3-propanediol. Using fed-batch fermentation, 109 g L^{-1} of industrial glycerol were converted into 58 g of 1,3-propanediol, 2.2 g of acetate and 6.1 g of butyrate per liter.

Keywords: *Clostridium butyricum*; glycerol; 1,3-propanediol

Introduction

Glycerol is a renewable resource found as a by-product of ethanolic fermentation of glucose or as a by-product of vegetable and animal fat processing. Many bacteria ferment glycerol into chemicals such as 1,3-propanediol, 2,3-butanediol, ethanol, acetic acid and lactic acid [7,11,12].

The Enterobacteriaceae such as *Klebsiella* are glycerol users, but these bacteria are classified as opportunistic pathogens [6,11,13]. However, several *Clostridium* species grow on glycerol and form 1,3-propanediol, including the non-pathogenic *Clostridium butyricum* [2,7,9]. Industrial glycerols from various fat feed-stocks may differ considerably with regard to their microbial conversion rate, in particular glycerol obtained through the transesterification process using rapeseed oil. Indeed, it can be toxic and we found no growth of several strains of *Clostridium butyricum* from three bacterial culture collections. The purpose of this study was to isolate strains of *C. butyricum* able to utilize industrial glycerol obtained from rapeseed oil.

Materials and methods

Organisms

Ten new strains of *C. butyricum* were isolated in this study. Strains IP 10034 and IP 3044 were purchased from the Institut Pasteur Collection (Paris, France), ATCC 859 and ATCC 19398 from the American Type Culture Collection (Rockville, MD, USA), and DSM 5431 was kindly provided by H Biebl (Braunschweig, Germany).

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Media and culture conditions

The strains were maintained on Reinforced Clostridial Medium (RCM, Oxoid Ltd, Basingstoke, UK) in Hungate tubes (18 × 150 mm). This medium was also used supplemented with 10 g L^{-1} glycerol as indicated in the text.

Precultures were performed in the medium described by H Biebl *et al* [1] that contained per liter: glycerol (Prolabo, Fontenay-sous-Bois, France, Ref 24387), 20 g; K_2HPO_4, 1 g; KH_2PO_4, 0.5 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 15 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; $CaCO_3$, 2 g; yeast extract, 1 g; trace element solution SL7, 2 ml [3]. Precultures were grown in 100-ml screw-capped bottles with rubber septa for syringe operation; the bottles were filled with 50 ml preboiled medium and sealed under nitrogen before autoclaving them.

Batch fermentations were performed in a 2-L fermentor (LSL-Biolafitte, Saint-Germain en Laye, France) containing 1 L of growth medium, with the glycerol concentration at 50 g L^{-1}, 'Commercial glycerol' was furnished by Prolabo; 'industrial glycerol' obtained through a transesterification process using rapeseed oil (Robbe, Diester Industrie, Compiègne, France) was used without purification and consisted of (g L^{-1}): glycerol, 650; Na_2SO_4, 40; non-glycerol organic material, 5; methanol, 1; H_2O, 300; various impurities, eg heavy metals, organosolv lignin, 4. Temperature was maintained at 32° C, pH was controlled at 7.0 by the automatic addition of 2 M KOH, and with an agitation of 100 rpm; in this case $CaCO_3$ was omitted.

Fed-batch cultures were started with 50 g L^{-1} glycerol, and after 20 h glycerol feeding (6 g L^{-1} h^{-1}) was continuously added to the fermentor by a variable special pump (Gilson model Minipuls 2, Villiers-le-Bel, France).

Isolation procedure

Mud samples were collected from a river, a stagnant pond, and a dry canal. Enrichment cultures were inoculated with 4 ml of anoxic muds plus vegetable scraps. The crude inocula were heat shocked at 80° C for 10 min and incubated at 34° C without shaking. After 1–5 days, dense cultures

Table 1 Characteristics of the isolates and comparison with reference strains of *C. butyricum*

Characteristics	New isolates										Reference strains		
	M1	M2	M3	E4	E5	F6	F7	F8	C10	C11	IP[a] 3044	IP[a] 10034	ATCC[b] 859
Indole produced	−	−	−	−	−	−	−	−	−	−	−	−	−
Starch pH	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolyzed	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin pH	+	w	+	+	+	+	+	+	+	w	+	+	+
Esculin hydrolyzed	+	+	+	+	+	+	+	+	+	+	+	+	+
Lecithinase produced	−	−	−	−	−	−	−	−	−	−	−	−	−
Lipase produced	−	−	−	−	−	−	−	−	−	−	−	−	−
Gelatin hydrolyzed	−	−	−	−	−	−	−	−	−	−	−	−	−
Acid from:													
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	w	+	+	+	+	+	+	+	+
Erythritol	−	w	w	+	+	+	+	w	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	w	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	−	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	w	+	w	w	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	w	+	w	+	w	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbose	w	+	+	−	+	+	+	−	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+
Milk reaction	c	c	c	c	c	c	c	c	c	c	c	c	c
Hemolysin	−	−	−	−	−	−	−	−	−	−	−	−	−+

[a]Strains tested in our laboratory
[b]*C. butyricum* as described by Anaerobe Laboratory Manual [11]
Symbols: + = reaction positive or pH of sugars below 5.5; − = reaction negative; w = weak reactive or pH of sugars 5.5–5.9; c = curd (for milk); −+ = 11–39% of strains positive

that produced gas bubbles were selected for isolation of glycerol-fermenting anaerobic organisms on solid media (1.5% agar). Inoculated plates were incubated in an anaerobic jar (BBL, Cockeysville, MD, USA) for 1–2 days. The isolated colonies were transferred into liquid media and the procedure was repeated to assure the purity of the cultures, ie until the bacterial cells appeared microscopically homogeneous. The three media contained glycerol.

Biochemical characteristics and growth measurement

The media and methods of Holdeman *et al* [10] were used for the biochemical tests. Growth was measured by optical density at 650 nm with a spectrophotometer (Shimadzu UV 160A double beam, Kyoto, Japan).

Analytical methods

Glycerol was determined enzymatically using the Boehringer Mannheim test kit.

Cell culture supernatant samples were analyzed for the fermentation products 1–3 propanediol, and acetic and butyric acids by gas chromatography by injecting supernatant medium from centrifuged cultures (12000 × *g* for 15 min) into an Intersmat IGC 121FL gas chromatograph equipped with a flame ionization detector. The injector and detector temperatures were 230° C. Separation took place in a 2-m (2-mm internal diameter) glass column packed with Chromosorb 101/80–100 mesh. Nitrogen was used as the carrier gas and *n*-butanol as internal standard. The column temperature was 170° C. The analysis of chromatographic data was carried out using an Intersmat ICR 1B Integrator (Delsi, Suresnes, France).

Glycerol and 1,3-propanediol tolerance

Growth inhibition was studied in Hungate tubes containing 9 ml RCM medium supplemented with glycerol (10 g L^{-1}), inoculated with 1 ml of cells in exponential phase and incubated at 34° C. A range of concentrations of glycerol or 1,3-propanediol were added except in the control tubes. The optical density at 650 nm was measured after 6 h in each tube. Inhibition (%) was determined from the following equation:

$$\frac{(OD_{c(t=6\,h)} - OD_{c(t=0)}) - (OD_{Ei(t=6h)} - OD_{Ei(t=0)})}{(OD_{c(t=6h)} - OD_{c(t=0)})} \times 100$$

where c = control tube and Ei = experiment ($i = 1,2,\ldots,x$)

Table 2 Batch fermentation of 5.3% commercial glycerol by strains of *C butyricum* from culture collections and by the ten new isolates

Clostridium strain	Glycerol used ($g L^{-1}$)	Products formed ($g L^{-1}$)			Butyrate	Conversion glycerol to 1,3 PD % (w/w)	Carbon recovery %
		Acetate	Butyrate	1,3-Propanediol	Acetate		
DSM 5431	48.8(1.6)	6.3(0.3)	1.3(0.1)	24.9(1.2)	0.2	51.0	87.1
IP 3044	47.0(1.5)	3.0(0.2)	4.2(0.3)	22.7(1.1)	1.4	48.0	87.0
ATCC 859	47.9(1.5)	3.3(0.2)	4.8(0.3)	22.0(1.1)	1.5	45.9	87.0
M1	49(1.8)	3.7(0.3)	4.6(0.2)	27.0(1.4)	1.2	55.0	98.0
M2	51.7(2.9)	4.2(0.3)	4.4(0.2)	26.3(1.3)	1	50.9	92.0
M3	48(1.7)	5.8(0.4)	2.2(0.1)	28.0(1.5)	0.4	58.0	99.0
E4	48.6(1.9)	2.8(0.2)	5.4(0.3)	24.8(1.2)	1.9	51.0	94.0
E5	52.6(2.1)	3.0(0.2)	5.5(0.4)	29.2(1.5)	1.8	55.5	97.7
F6	46.7(2.3)	3.2(0.3)	4.0(0.2)	23.4(1.2)	1.2	50.1	89.0
F7	48.3(1.6)	1.4(0.1)	5.8(0.3)	25.1(1.3)	4.1	52.0	92.5
F8	49.3(1.9)	3.5(0.2)	4.8(0.2)	28.1(1.4)	1.4	56.0	99.0
C10	53(1.9)	1.6(0.1)	6.7(0.4)	28.5(1.4)	4.2	55.0	96.0
C11	48.4(1.8)	1.5(0.1)	7.2(0.4)	25.0(2.2)	4.8	51.0	98.3

(): Standard deviation of three determinations

Table 3 Batch fermentation of 5.3% 'industrial' glycerol by four new isolates

Clostridium strain	Glycerol used ($g L^{-1}$)	Products formed ($g L^{-1}$)			Butyrate	Conversion glycerol to 1,3 PD % (w/w)	Carbon recovery %
		Acetate	Butyrate	1,3-Propanediol	Acetate		
M2	21.0 (1.3)	1.8 (0.2)	1.6 (0.2)	9.5 (0.6)	0.9	45.0	83.9
M3	53.0 (2.1)	3.0 (0.3)	5.5 (0.3)	25.0 (1.2)	1.8	47.0	87.5
E4	44.0 (2.1)	1.2 (0.1)	6.5 (0.4)	20.0 (1.1)	5.4	45.0	90.0
E5	53.0 (2.3)	2.2 (0.2)	6.1 (0.4)	27.0 (1.4)	2.8	51.0	92.0

Cultures were analyzed after 24 h of growth
(): Standard deviation of three determinations

Table 4 Fed-batch cultures by two new isolates[a]

Strain	Type of glycerol	Glycerol used ($g L^{-1}$)	Products formed ($g L^{-1}$)			Butyrate	Conversion glycerol to 1,3 PD % (w/w)	Carbon recovery %
			Acetate	Butyrate	1,3 PD	Acetate		
E4	commercial	108 (2.5)	2.6 (0.2)	14 (0.8)	57.9 (2.5)	5.4	53.6	95
	industrial	96.0 (3)	1.4 (0.1)	13.8 (0.7)	50.3 (2.1)	9.8	47.4	95.7
E5	commercial	122 (3.5)	2 (0.15)	15.9 (0.8)	65.6 (3)	8	54.0	95
	industrial	109.2 (4)	1.6 (0.15)	14.6 (0.7)	58.4 (2.8)	9	53.5	95

[a]The initial concentration of 'commercial' or 'industrial' glycerol was 50 $g L^{-1}$, then after 20 h incubation glycerol feeding was realized at a constant rate of 6 g per h. The final fermentation volume was 1.3 L. Cultures were analyzed afer 48 h of fermentation
(): Standard deviation of three determinations

Results

Isolation of ten new strains

The enrichment and selection procedures yielded pure cultures of ten obligately anaerobic, spore-forming, mesophilic and glycerol-fermenting bacteria. The cells were Gram-positive, rod-shaped with subterminal spores. The differential characteristics indicated that the isolates can be placed in the genus *Clostridium*, among the saccharolytic species. Using the test system of Holdeman *et al* [10] (Table 1), the ten isolates could be classified as *C. butyricum*; the strains were distinguished from *C. acetobutylicum* by their failure to liquefy gelatin, from *C. beijerinckii* by their ability to grow in a mineral medium (GMB) and from *C. pasteurianum* due to their different pattern of use of carbon source utilization. The ten isolates proved to be identical in almost all characteristics.

Batch cultures

Fermentation of 'commercial' glycerol: The ten new isolates and the five strains from culture collections were screened for growth on glycerol (5.3%) and for synthesis of 1,3-propanediol. In our batch conditions, among the strains from collections, *C. butyricum* IP 10034 and ATCC 19398 did not grow on glycerol. As shown in Table 2, all other strains ie *C. butyricum* IP3044, ATCC 859, DSM

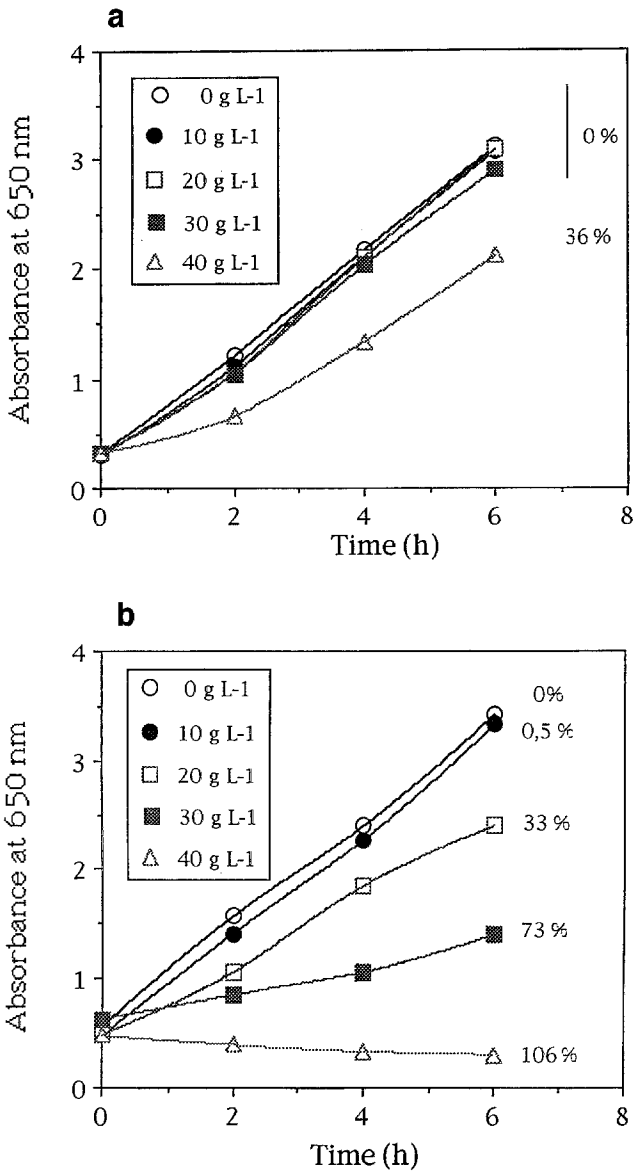

Figure 1 Effect of various concentrations of 1,3-propanediol on cellular growth. Cultures were incubated in Hungate culture tubes containing RCM medium supplemented with 10 g L^{-1} glycerol. 0, 10, 20, 30, 40 g L^{-1} refer to the concentration of the additional 1,3 propanediol. Percentages correspond to the inhibition rate. (a) = *C. butyricum* E5; (b) = *C. butyricum* DSM 5431

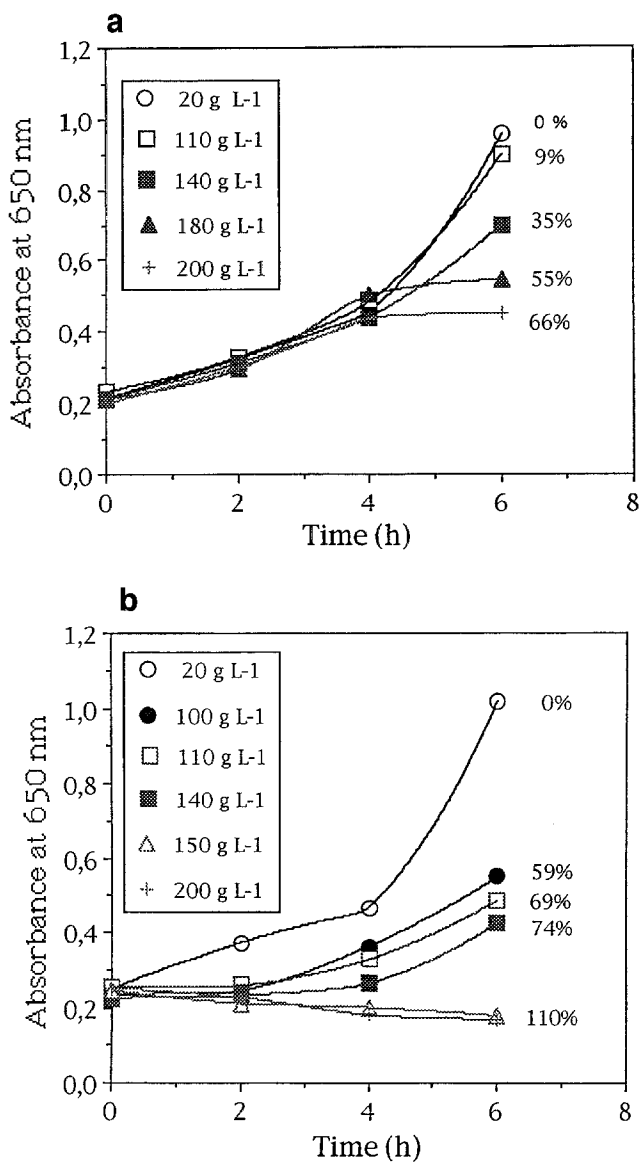

Figure 2 Effect of glycerol on cellular growth. Cultures were incubated in Hungate culture tubes in RCM medium. 20, 100, 110, 140, 150, 180, 200 g L^{-1} refer to the concentration of the additional glycerol. Percentages correspond to the inhibition rate. (a) = *C. butyricum* E5; (b) = *C. butyricum* DSM 5431

5431 as well as the ten new isolates almost completely fermented the glycerol and produced 1,3-propanediol as a major fermentation product with concomitant production of acetic and butyric acids. Carbon recoveries varied between 87–99%, and from 45 to 58% (w/w) of the glycerol was converted to 1,3-propanediol while the ratios of butyric acid/acetic acid were found in the range of 0.2 to 4.8. Concerning the butyrate/acetate ratios, three kinds of strains were found, those which produced (i) more acetate than butyrate (eg DSM 5431), (ii) the same quantities (eg M2), (iii) more butyrate than acetate (C11).

Fermentation of the 'industrial' glycerol: Strains obtained from bacterial culture collections were unable to grow on 'industrial' glycerol and, among the ten isolates, only four strains grew on this carbon source (Table 3). Three strains of *Clostridium*, E4, M3, E5, efficiently used the industrial glycerol, especially the latter two strains. For these three strains industrial glycerol had a slight decrease in the percentage conversion into 1,3-propanediol. Moreover butyrate production increased for all strains.

Fed batch cultures

To alleviate the inhibitory effect of high glycerol concentrations, a fed-batch supply of glycerol was evaluated in order to lessen the inhibitory effects. As shown in Table 4, good production of 1,3-propanediol proceeded in fed batch cultures. For *Clostridium* strain E4, glycerol conversion (47.4%) on industrial glycerol was lower than that obtained on commercial glycerol (53.6%), but for *Clostridium* strain E5 the same values (53–54%) were obtained for both sub-

strates. The ratios of butyric acid/acetic acid were strongly influenced by the mode of glycerol supply: more butyric acid was produced in fed-batch (Table 4) than in batch fermentation (Table 3).

Inhibitory effects on growth of C. butyricum *DSM 5431 and* C. butyricum *E5*

Isolate *C. butyricum* E5 was far more resistant to inhibitory substances than the strains from culture collections. To evaluate the potential of *C. butyricum* E5 to survive at high concentrations of 'inhibitor', its growth was monitored in batch culture to which glycerol and 1,3-propanediol were added in increasing concentrations (Figures 1a and 2a). The performances of *C. butyricum* strain E5 were compared to the well known strain *C. butyricum* DSM 5431 (Figures 1b and 2b). 1,3-propanediol was more toxic for *Clostridium* DSM 5431 (Figure 1). At 40 g L^{-1} of 1,3-propanediol the inhibition of growth for *Clostridium* E5 was 36%, whereas it was 106%, due to a total inhibition plus cell lysis, for *Clostridium* DSM 5431. At 200 g L^{-1} of glycerol, growth was totally inhibited for *Clostridium* DSM 5431 while 66% inhibition was observed for *Clostridium* E-5 (Figure 2).

Discussion

A variety of strains of *C. butyricum* grew on purified glycerol (Table 2). Among five strains from culture collections and ten new isolates, only two strains failed to use this carbon source. If glycerol was used, the positive strains synthesized 1,3-propanediol as a major product with good yields and final 1,3-propanediol concentrations. Since the reduction of glycerol to 1,3-propanediol after dehydration to 3-hydroxypropionaldehyde produces no ATP, the concomitant formation of acetate and butyrate is needed [4]. Significant variations in the butyric acid/acetic acid ratios were observed depending on the strains used, suggesting different regulatory mechanisms of the redox equilibrium state at the level of the ferredoxin oxidoreductase activities and the hydrogenase [4,9].

Glycerol produced by cleavage of natural fats can be converted by microorganisms to 1,3-propanediol without further purification [5]. However, industrial glycerol obtained through the transesterification process leads to a toxic substrate, which cannot be used by any of the *C. butyricum* strains from culture collections we tested. Product inhibition in the clostridial glycerol fermentation is much more an effect of 1,3-propanediol than of butyric and acetic acid—although the latter are more toxic to the microorganisms—but occurs at concentrations too low to affect the cells appreciably [14]. Inhibition by individual products has been determined in *Clostridium butyricum* E5 and also *C. butyricum* DSM-5431 which is well known [1,2,5,8] and hence which we used as a representative strain. The results obtained showed clearly a greater resistance for the new isolate which was able, in fed-batch fermentation, to convert 109 g L^{-1} of raw glycerol into 58 g of 1,3-propanediol, whereas the *Clostridium* DSM strain converted 108 g of purified glycerol into 55 g of 1,3-propanediol [8].

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