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Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains

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Summary. Glycerol-fermenting anaerobes were enriched with glycerol at low and high concentrations in order to obtain strains that produce 1,3-propanediol. Six isolates were selected for more detailed characterization; four of them were identified as *Citrobacter freundii,* one as *Klebsiella oxytoca* and one as *K. pneumoniae*. The *Citrobacter* strains formed 1,3-propanediol and acetate and almost no by-products, while the *Klebsiella* strains produced varying amounts of ethanol in addition and accordingly less 1,3-propanediol. Enterobacterial strains of the genera *Enterobacter, Klebsiella*, and *Citrobacter* from culture collections showed similar product patterns except for one group which formed limited amounts of ethanol, but no propanediol. Seven strains were grown in pH-controlled batch cultures to determine the parameters necessary to evaluate their capacity for 1,3-propanediol production. *K. pneumoniae* DSM 2026 exhibited the highest final concentration (61 g/l) and the best productivity (1.7 g/l h) whereas *C. freundii* Zu and K2 achieved only 35 g/l and 1.4 g/l h, respectively. The *Citrobacter* strains on the other hand gave somewhat better yields which were very close to the theoretical optimum of 65 mol %.

Introduction

As a glycol, 1,3-propanediol can principally be utilized as a monomer for the synthesis of polyesters and polyurethanes. The polymers on the basis of 1,3-propanediol are considered to exhibit better product properties and greater light stability than those produced from 1,2-propanediol, butanediols, or ethylene glycol (Elm et al. 1980). 1,3-Propanediol is synthesized at present in only small amounts from acrolein. The relatively high price, which is due to costly distillation, has prevented its application in polymer industries up to now. If 1,3-propanediol could be produced more cheaply by a fermentation process its use for the synthesis of plastics with specific properties might be envisaged. Glycerol as the chemical feedstock could be supplied as diluted raw glycerol, which is released from fat saponification for soap and detergent manufacture.

Fermentation of glycerol occurs rarely in rather different bacterial groups. It is oxidized via dihydroxyacetone to glyceraldehyde-3-phosphate and further via pyruvate to acetate, formate, CO_2 and other products. The reducing equivalents may be transferred to an external electron acceptor or to the dehydration product of glycerol, 3-hydroxypropionaldehyde. The final product, 1,3-propanediol, is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion.

Among the Enterobacteriaceae, anaerobic growth with glycerol and formation of 1,3-propanediol is used as a character for identification of the genus *Citrobacter*, but is also found in strains of *Klebsiella* (Toraya et al. 1980). The products of glycerol oxidation arise from pyruvate cleavage to acetyl coenzyme A and formate, resulting in excretion of ethanol, acetate, CO_2 and H_2 (Mickelson and Werkman 1940). Streekstra et al. (1987) have recently investigated the energetic aspects of glycerol fermentation to 1,3-propanediol in chemostat cultures of *K. pneumoniae*.

Several *Clostridium* species also form 1,3-propanediol, together with butyric and acetic acid and some butanol or lactic acid (Nakas et al. 1983; Forsberg 1987). Some lactic acid bacteria form 1,3-propanediol from glycerol if a growth and energy substrate is present in the medium (Schütz and Radler 1984). Recently strictly anaerobic non-spore-forming, glycerol-utilizing rods have been described which ferment glycerol in a disproportionation reaction to 1,3-propanediol and 3-hydroxypropionic acid (Schink and Stieb 1983; Stieb and Schink 1984). Nothing is known about their fermentation capacity.

Suitable organisms for a glycerol fermentation process might be looked for among established strains of the genera *Citrobacter*, *Klebsiella*, or *Clostridium*. However, up to now no effort to investigate the natural

potential for 1,3-propanediol-producing organisms has been published. It was the purpose of this study to obtain the bacteria representing this fermentation type from anaerobic enrichment cultures, to compare them with existing glycerol-fermenting strains, and to evaluate them for technical applications.

Materials and methods

Media and growth conditions. Enrichment cultures from sediments and sewage sludge with 0.09% glycerol were performed in a bicarbonate-buffered, sulphide-reduced mineral medium (Schink and Pfennig 1982). Enrichment cultures with 2% glycerol, precultures, and cultures for preliminary tests were grown in 100-ml screw-capped bottles with rubber septa for syringe operation. They were filled with 30 ml preboiled medium and sealed under nitrogen before autoclaving. The medium contained in g/l deionized water: glycerol, 20 g; K_2HPO_4, 3.4 g; KH_2PO_4, 1.3 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 5 mg; yeast extract, 1 g; trace element solution SL 7 (Biebl and Pfennig 1981), 2 ml; $CaCO_3$, 2 g. The incubation temperature was 30° C.

For growth and product formation experiments a 1-l fermentor of BCC, Göttingen, FRG, was used. It was equipped with a control unit to adjust the pH to 7.0 and the agitation speed to 150 rpm. The temperature was maintained at 32° C using an external thermostat. Slight gassing with purified nitrogen provided anaerobic conditions. The medium for fermentor cultures was amended to contain only 1 g K_2HPO_4 and 0.5 g KH_2PO_4, and no $CaCO_3$.

Isolation. Pure cultures were obtained by repeated application of the agar shake culture method (Pfennig 1978) using screw-capped test tubes which were gassed with N_2. Purified isolates were kept semi-aerobically on agar slants.

Analytical techniques. The fermentation products were separated and determined gas chromatographically on a Chromosorb 101 column of 1 m length installed in a Packard instrument, Model 408A (Chrompack, Frankfurt/M, FRG). The column temperature was programmed from 150° C to 220° C in several steps; the carrier gas was nitrogen. Isobutanol was used as internal standard.

Glycerol appeared after the fermentation products at the end of the chromatograms as a broad peak which allowed a rough estimation of its concentration. Quantitative determination was performed enzymatically via glycerokinase and L-lactate dehydrogenase using the test kit and instructions of Boehringer, Mannheim, FRG. D-Lactate was also measured by a Boehringer test operating with D-lactate dehydrogenase and glutamate-pyruvate transaminase.

Optical density was measured at 650 nm in a 1-cm cuvette. Cell dry weights were obtained from washed centrifuged pellets dried at 80° C for 24 h.

For identification of isolated Enterobacteriaceae strains the Enterotube II test kit of F. Hoffmann La Roche (Basel, Switzerland) was applied.

Results

Enrichment and isolation

Enrichment with 0.09% glycerol. Half-filled 120-ml serum bottles were inoculated each with 5 ml of anoxic black sediment or sewage sludge samples, and closed under a N_2/CO_2 mixture (90%/10%) with butyl rubber stoppers. Growth and gas production started after 1–5 days, and subsequent transfers grew up within 1–2 days. Purification of the prevalent bacteria by agar shake culture yielded the strains Zu from a sugar factory sewage sludge (Nörten-Hardenberg, FRG), Gö from the municipal sewage treatment plant in Göttingen, FRG, and Lin from a polluted creek near Hannover, FRG.

Enrichment with 2% glycerol. Four anaerobic culture bottles were inoculated with samples of garden compost, pond sediment and sludge (fresh and activated) of the Wolfenbüttel sewage plant (FRG). After 1 or 2 days, growth and 1,3-propanediol formation occurred in each of them. From the sewage culture, strain Bs2e was isolated and corresponded to strain Lin in cell shape and colony size. From the compost was obtained strain K2 and from the pond sediment strain T3, which both corresponded to strains Zu and Gö. All six strains produced 1,3-propanediol. A third group of isolates which grew weakly and formed only ethanol in low amounts was not further regarded.

Although conditions were anaerobic throughout we did not obtain clostridia from any of the enrichments.

Identification

All six isolates obtained were rod-shaped. Strains Lin and Bs2e had rather large, but somewhat varying cells of $0.8–1.4 \times 2–6$ μ in size when grown on glycerol and were immotile. The other strains measured only $0.6–0.9 \times 1.3–4$ μ and were strikingly motile. Negative contrast electron micrographs revealed peritrichous flagellation.

All strains grew aerobically and gave a negative oxidase reaction. The products of glucose fermentation were predominantly 2,3-butanediol and ethanol with the immotile strains and acetic and lactic acid with the motile ones. A biochemical test system allowed classification of the immotile strains as *Klebsiella* sp. and the motile ones as *Citrobacter freundii.* Strain Lin was identified as *K. oxytoca,* and strain Bs2e as *K. pneumoniae* because of a positive indole reaction. The determination of strain Bs2e was substantiated by additional tests, i.e. a positive fecal coliform reaction, no growth at 10° C and no utilization of melizitose and gentisate.

Fermentation products from glycerol

Isolates. Table 1 shows the fermentation patterns of the isolates when grown in a 2% glycerol medium with $CaCO_3$ buffering. While the *Klebsiella* strains produced predominantly ethanol (Lin) or both ethanol and acetate (Bs2e) in addition to 1,3-propanediol, the *Citrobacter* strains fermented glycerol almost entirely to 1,3-propanediol and acetate.

Except for strain Bs2e the fermentation ceased after consumption of 80–90 mmol/l glycerol at a pH of about 4.8. Glycerol was completely used up when the culture pH was regularly readjusted to 7.0 (not shown). When the growth temperature was raised to 37° C, fer-

Table 1. Anaerobic utilisation of glycerol by isolates in a $CaCO_3$-buffered medium

Species	Strain	Glycerol consumed (mmol/l)	Products formed (mmol/l)			
			1,3-Propanediol	Ethanol	Acetic acid	Lactic acid
Klebsiella oxytoca	Lin	92	31	24	3	++
K. pneumoniae	Bs2e	158	81	19	12	+
Citrobacter freundii	Zu	86	41	1	12	+
C. freundii	Gö	91	42	1	12	+
C. freundii	T3	76	33	1	11	+
C. freundii	T2	79	43	3	13	+

Table 2. Anaerobic utilization of glycerol by selected Enterobacteriaceae strains from culture collections

Species	Strain	Glycerol consumed (mmol/l)	Products formed (mmol/l)			
			1,3-Propanediol	Ethanol	Acetic acid	Lactic acid
Enterobacter aerogenes	DSM 30053	53	Trace	36	1	1
K. terrigena	DSM 2687	51	Trace	51	1	1
K. trevisani	DSM 2688	71	Trace	66	1	1
K. planticola	DSM 3060	73	0	72	1	0
K. planticola	IAM 1133	108	21	62	3	4
K. oxytoca	NRCC 3006	90	55	39	11	11
K. pneumoniae	DSM 2026	108	73	4	8	12
C. freundii	DSM 30039	53	33	1	12	0
C. freundii	DSM 30040	57	32	1	12	0

Glycerol was not fermented by *E. cloacae* DSM 30054 and DSM 30056 and by *Bacillus polymyxa* DSM 36 and NRCC 9053

mentation was markedly slower in *K. oxytoca* Lin and *C. freundii* Zu; *C. freundii* Gö did not grow at 37° C.

Collection strains. Enterobacterial strains as well as two bacilli from culture collections exhibited rather different fermentation patterns (Table 2). Two strains of *Enterobacter cloacae* and the two bacilli did not ferment glycerol at all. One *Enterobacter* and three *Klebsiella* strains of different species formed limited amounts of ethanol but no propanediol. Three other *Klebsiella* strains formed propanediol in addition to ethanol and acetate, the proportion of which varied considerably. The two *C. freundii* strains behaved as the isolated ones, 1,3-propanediol and acetate being almost the only products.

PH-controlled glycerol fermentations

Three *Klebsiella* and four *Citrobacter* strains were selected to be grown in pH-controlled fermentor cultures with 2% glycerol. The final results listed in Table 3 do

Table 3. Fermentation of 2% glycerol in a 1-l fermentor at pH 7.0

Species	Strain	Glycerol consumed (mmol/l)	Fermentation time (h)	Cell mass (g/l)	Products formed (mmol/l)				Product recovery (mol/100 mol glycerol)		Overall propanediol productivity (g/l·h)
					1,3-Propanediol	Ethanol	Acetic acid	Lactic acid	Overall	Propanediol	
K. oxytoca	NRCC 3006	220	12[a]	1.2	78	79	35	13	93	36	0.49
K. oxytoca	Lin	224	12	1.6	87	82	28	12	93	38	0.55
K. pneumoniae	DSM 2026	237	7[a]	1.6	123	24	58	2	87	52	1.33
C. freundii	DSM 30039	247	20	0.8	173	5	54	5	96	70	0.65
C. freundii	Gö	212	18	0.8	138	11	40	15	96	65	0.58
C. freundii	Zu	248	13	1.2	158	6	65	4	94	64	0.92
C. freundii	K2	252	14	1.0	163	19	99	5	92	65	0.88

[a] Growth temperature 37° instead of 32° C

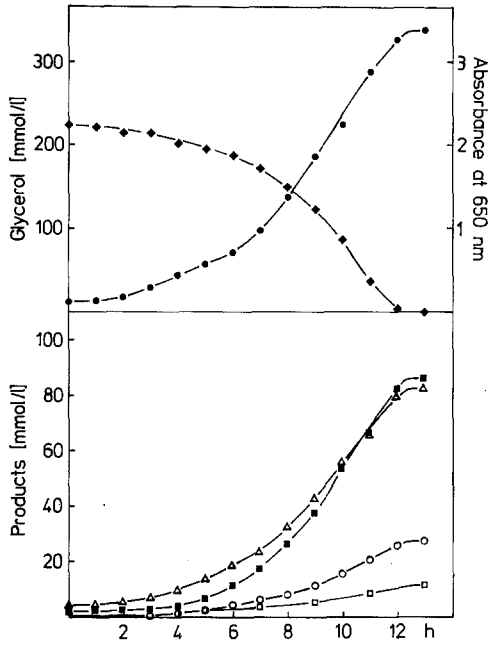

Fig. 1. Fermentation of 2% glycerol by *Klebsiella oxytoca* Lin at 32° C and pH 7.0: ●, absorbance; ◆, glycerol; ■, 1,3-propanediol; △, ethanol; ○, acetic acid; □, lactic acid

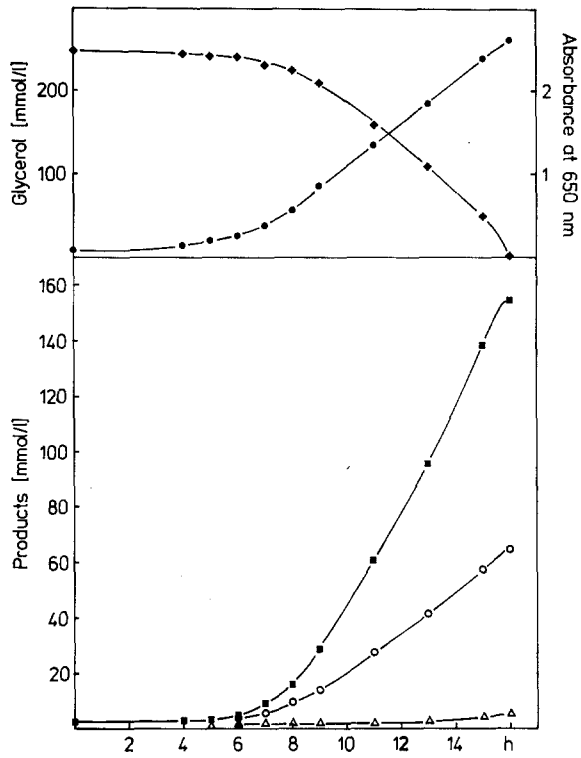

Fig. 2. Fermentation of 2% glycerol by *Citrobacter freundii* Zu at 32° C and pH 7.0: symbols as in Fig. 1

not differ too much from those of the bottle cultures (Tables 1 and 2), but they permit a better recognition of the fermentation balance. The two *K. oxytoca* strains behaved very similarly: they formed a relatively high amount of ethanol and consequently less 1,3-propanediol and acetic acid. In the *Citrobacter* cultures there was some ethanol and lactic acid, too, at the end of the fermentation, but acetic acid and 1,3-propanediol were by far the predominating products. *K. pneumoniae* DSM 2026 exhibited a similar product composition, but the propanediol yield was markedly lower than with *Citrobacter.*

The fermentation time for 2% glyerol was somewhat shorter with *K. oxytoca* than with *Citrobacter*, but due to the higher yields, the overall productivity for propanediol was superior with the *Citrobacter* strains. Highest productivity was achieved by *K. pneumoniae* DSM 2026 because of its unusually high fermentation rate.

In Figs. 1 and 2 the kinetics of growth, substrate utilization and product formation are shown for one strain of each group. Cultures of *Citrobacter* never grew without a delay of 3–6 h, during which time the cells elongated considerably. The exponential growth phase lasted about 6 h for all strains and was followed by a phase of linear growth. There was no shift of product formation with the growth phases; all products increased in a fixed proportion.

As shown in Table 4 5% glycerol was also completely fermented (the two slow-growing *Citrobacter* strains were not included). The product composition did not change with *Citrobacter*; with *Klebsiella* the proportions shifted somewhat from ethanol to propanediol and acetate. The calculated 1,3-propanediol productivity was higher with 5% than with 2% glycerol in all five cultures.

Table 4. Fermentation of 5% glycerol in a 1-l fermentor at pH 7.0

Species	Strain	Glycerol consumed (mmol/l)	Fermentation time (h)	Cell mass (g/l)	Products formed (mmol/l)				Product recovery (mol/100 mol glycerol)		Overall propanediol productivity (g/l·h)
					1,3-Propanediol	Ethanol	Acetic acid	Lactic acid	Overall	Propanediol	
K. oxytoca	NRCC 3006	543	25[a]	3.0	223	100	68	32	78	41	0.68
K. oxytoca	Lin	552	20	2.1	239	98	44	22	73	43	0.91
K. pneumoniae	DSM 2026	553	12[a]	3.5	294	23	88	32	79	53	1.68
C. freundii	Zu	575	21	1.8	370	11	134	13	92	64	1.33
C. freundii	K2	544	19	1.9	342	30	112	2	87	63	1.37

[a] Growth temperature 37° instead of 32° C

A glycerol concentration of 10% was used only by *K. pneumoniae* DSM 2026, which also fermented 12% glycerol. With the other strains, best glyerol utilization was obtained with *C. freundii* K2 with 63 g/l, while *K. oxytoca* Lin did not grow at all. Strain K2 fermented 7%, 9% and 10% glycerol with about the same result indicating that the maximum fermentation capacity is mainly determined by product inhibition.

Discussion

Among the strains isolated from enrichment cultures with glycerol and the collection strains screened for glycerol utilization, different fermentation types were recognized. One group including an *Enterobacter* and several *Klebsiella* strains as well as some unidentified isolates, is characterized by formation of ethanol as the only product and by limited glycerol turnover. Although the fermentation balance is theoretically compensated in the case of ethanol formation from glycerol, there are some reduction equivalents released during cell mass formation which are obviously transferred to external electron acceptors such as yeast extract components present in the medium (Thimann 1963). The other glycerol utilizers transfer the surplus reducing equivalents to further glycerol molecules releasing 1,3-propanediol as a fermentation product. This type is represented by a number of *Klebsiella* species on the one hand and *Citrobacter* on the other.

The majority of the *Klebsiella* strains forms greater proportions of ethanol and accordingly less 1,3-propanediol while *Citrobacter* performs an almost pure 1,3-propanediol/acetic acid fermentation of glycerol. A very low final ethanol and fairly high 1,3-propanediol content is also present in cultures of *K. pneumoniae* strain DSM 2026 (= NCTC 418) and strain Bs2e. However, the ethanol proportion increases with DSM 2026 under conditions of energy substrate limitation (Streekstra et al. 1987; unpublished results), whereas glycerol fermentation of *Citrobacter* seems to be almost uninfluenced by the culture conditions.

No organisms have been found which form ethanol as the main product in addition to a small amount of 1,3-propanediol just to compensate for the redox level difference between glycerol and cell material. This means that propanediol is always produced concomitantly with acetic acid. Streekstra et al. (1987) found the minimum of 1,3-propanediol excretion which appeared under glycerol limitation in cultures of *K. pneumoniae* DSM 2026 to remain at a relatively high level, and they inferred that the two enzymes involved in glycerol reduction have a higher affinity to the substrate than the oxidizing enzyme.

Clostridia were not enriched from the cultures described, but they invariably appeared when the samples were pasteurized prior to inoculation (Widdel, personal communication, unpublished observations). This might indicate a selective advantage for enteric bacteria in the habitats considered or an absence of vegetative *Clostridium* cells in the samples.

For evaluation of a fermentation process for technical application one has to consider the yield of the product desired, the substrate turnover rate and the final product concentration, i.e. the tolerance of the organism towards its products. Among the investigated strains, the highest yield of 1,3-propanediol was achieved by *Citrobacter* which approached the theoretical maximum:

$$\text{Glycerol} \rightarrow 0.33 \text{ acetate} + 0.67 \text{ 1,3-propanediol} + 0.33\ H_2 + 0.33\ CO_2.$$

If the cell mass production, which was estimated from the batch cultures to be around 5% of the glycerol utilized, is taken into account the 1,3-propanediol yield does not change significantly due to the redox level difference between glycerol and cell mass:

$$\text{Glycerol} \rightarrow 0.3 \text{ acetate} + 0.65 \text{ 1,3-propanediol} + 0.3\ H_2 + 0.3\ CO_2 + 0.05\ [C_4H_8O_3].$$

However, depending on the strain and the culture conditions, the by-products in *Citrobacter* cannot be neglected and vary from 2% to 7% for ethanol and 1% to 8% for lactic acid.

The 1,3-propanediol yield of *K. pneumoniae* was lower than that of *Citrobacter* and amounted only to 0.53 mol/mol glycerol for strain DSM 2026 in contrast to 0.65 mol for *Citrobacter*. However, due to its rapid fermentation the resulting productivity of *K. pneumoniae* for propanediol is considerably higher. As DSM 2026 in addition exhibits a product tolerance which is twice as high as with *Citrobacter*, this strain must be regarded as the most suitable one for the development of a 1,3-propanediol fermentation process at present. However, since *K. pneumoniae* is regarded as an opportunistic pathogen and thus may give rise to safety discussions the classical "trimethyleneglycol" formers of the genus *Citrobacter* should be kept in mind for future research activities in fermentative 1,3-propanediol production.

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