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# Effect of glucose on glycerol metabolism by *Clostridium butyricum* DSM 5431

**S. Abbad-Andaloussi, J. Amine[1], P. Gerard and H. Petitdemange**

*Laboratoire de Chimie Biologique I, Université Henri Poincare, Nancy, Vandœuvre-lès-Nancy Cédex, France and [1]Laboratoire de Biochimie, Faculté des Sciences, Université Chouaib Doukkali, El jadida, Morocco*

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S. ABBAD-ANDALOUSSI, J. AMINE, P. GERARD AND H. PETITDEMANGE. 1998. The levels of 1,3-propanediol dehydrogenase and of the glycerol dehydrogenase in *Clostridium butyricum* grown on glucose–glycerol mixtures were similar to those found in extracts of cells grown on glycerol alone, which can explain the simultaneous glucose–glycerol consumption. On glycerol, 43% of glycerol was oxidized to organic acids to obtain energy for growth and 57% to produce 1,3-propanediol. With glucose–glycerol mixtures, glucose catabolism was used by the cells to produce energy through the acetate–butyrate production and NADH, whereas glycerol was used chiefly in the utilization of the reducing power since 92–93% of the glycerol flow was converted through the 1,3-propanediol pathway. The apparent $K_m$s for the glycerol dehydrogenase was 16-fold higher for the glycerol than that for the glyceraldehyde in the case of the glyceraldehyde-3-phosphate dehydrogenase and fourfold higher for the $NAD^+$, providing an explanation for the shift of the glycerol flow toward 1,3-propanediol when cells were grown on glucose–glycerol mixtures.

## INTRODUCTION

*Clostridium butyricum* is known as a classical acid producer and usually ferments glucose to butyrate, acetate, carbon dioxide and molecular hydrogen (Jungermann *et al*. 1973). When *Cl. butyricum* is growing on glycerol, this substrate is oxidized to dihydroxyacetone (DHA) and subsequently phosphorylated to yield DHA-phosphate, or it is dehydrated to 3-hydroxypropionaldehyde (3-HPA); the latter compound is then reduced to 1,3-propanediol (1,3-PD) (Biebl *et al*. 1992; Dabrock *et al*. 1992).

When glycerol and glucose are compared as carbon sources for growth, they show a number of differences in catabolism (Abbad-Andaloussi *et al*. 1996). In glucose-grown cells, the difference between the specific production rate of NADH and the specific consumption rate indicates an excess of NADH which was reoxidized by the NADH-ferredoxin reductase and the reduced ferredoxin (fd) reoxidized by hydrogenase (Abbad-Andaloussi *et al*. 1996). In contrast, growth on glycerol indicates that the NADH produced by glycerol dehydrogenase (GDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is not sufficient for 1,3-PD formation and that part of the reduced fd produced by the pyruvate-fd oxidoreductase is reoxidized by fd-$NAD^+$ reductase activity to produce NADH (Abbad-Andaloussi *et al*. 1996). The objective of this work was to obtain a more precise understanding of the metabolic aspect of batch fermentation of glucose–glycerol mixtures by *Cl. butyricum* DSM 5431 and to elucidate the co-ordination of expression of the various enzymatic activities of the glucose and glycerol pathways.

*Correspondence to: H. Petitdemange, Laboratoire de Chimie Biologique I, Université Henri Poincare, Nancy I, B.P. 239, 54506 Vandœuvre-lès-Nancy Cédex, France.*



## MATERIALS AND METHODS

### Chemicals

All chemicals were reagent grade. Enzymes and coenzymes were obtained from Sigma Chimie (St Quentin Fallavier, France). All gases used were purchased from Air Liquide (Paris, France).

### Organism and medium

*Clostridium butyricum* DSM 5431 was used. The spores of the strain were stored at 4 °C in Hungate tubes in Reinforced Clostridial Medium (RCM; Oxoid Ltd, Basingstoke, UK).

For inoculum preparation, spores were transferred to RCM medium, heat shocked at 80 °C for 10 min and incubated at 35 °C under anaerobic conditions in Hungate tubes.

The preculture medium contained the following components ($l^{-1}$ distilled water): glycerol, 20 g; $K_2HPO_4$, 1·0 g; $KH_2PO_4$, 0·5 g; $(NH_4)_2SO_4$, 2·0 g; $MgSO_4.7H_2O$, 0·2 g; $CaCl_2.2H_2O$, 15 mg; $FeSO_4.7H_2O$, 5 mg; $CaCO_3$, 2·0 g; yeast extract, 1·0 g; trace element solution SL7 (Biebl and Pfenning 1982) 2 ml and glucose or glycerol, 10 g. 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.

The growth temperature was 32 °C. This medium, without $CaCO_3$ and with different concentrations of glucose, glycerol or glucose–glycerol mixtures, was used as the culture medium. An exponential culture grown in the preculture medium was used as inoculum (10%, v/v) in a fermenter (LSL-Biolafitte, St Germain en Laye, France; 2 litre growth vessel with a 1 litre working volume). The culture was stirred at 100 rev $min^{-1}$. The pH was maintained at 7·0 by the automatic addition of 2 mol $l^{-1}$ KOH, and anaerobic culture conditions were maintained by sparging with nitrogen at a flow rate of 0·1 volume of gas per volume of liquid per minute.

For determination of specific rates of substrate utilization, the anaerobic fermentations were done in flasks with 200 ml of liquide-volume and with glucose, glycerol or glucose–glycerol mixtures.

## Analytical methods

Glycerol was determined enzymatically by glycerol kinase, pyruvate kinase and L-lactate dehydrogenase enzymes using the test kit and instructions of Boehringer (Mannheim, Germany). Glucose was determined using glucose oxidase (glucose diagnostic kit No. 510; Sigma). Concentrations of 1,3-PD, acetic and butyric acids were determined by a gas chromatograph (Intersmat IGC 121 FL) equipped with a flame ionization detector. Separation took place in a glass column (2 m × 2 mm internal diameter) packed with chromosorb 101/80-100 mesh. Nitrogen was used as the carrier gas and n-butanol as the internal standard. The temperature of the column was 170 °C and data were analysed with an Intersmat ICR IB Integrator.

The cell concentrations were estimated as cell dry weight using a predetermined correlation between absorbance at 650 nm (Shimatzu UV160A double beam spectrophotometer, Kyoto, Japan) and cell dry weight.

## Preparation of cell-free extracts

Cells grown on 60 g $l^{-1}$ glycerol from a fermenter were centrifuged at 12 000 *g* for 15 min; after washing (except for hydrogenase activity) cells were resuspended in Tris buffer sparged with nitrogen (50 mmol $l^{-1}$ Tris-HCl; 2·0 mmol $l^{-1}$ DL-dithiothreitol; 0·1 mmol $l^{-1}$ $MnSO_4$; pH 7·4). The cells were sonicated at 2 °C for 20 s at a frequency of 20 kHz, followed by a 60 s pause (150 W ultrasonic disintegrator; MSE, UK); this cycle was repeated four times. The supernatant fluid was collected from the cell lysate by centrifugation at 12 000 *g* for 20 min at 4 °C. At each step, extracts were maintained under a nitrogen atmosphere.

Protein concentrations of cell extracts were determined according to the Bradford (1976) method, using crystalline bovine serum albumin as the standard.

## Enzyme assays

Glycerol dehydrogenase (EC 1.1.1.6) and 1,3-PD dehydrogenase (EC 1.1.1.202) activities were measured by the linear increase in absorbance at 340 nm ($A_{340}$) produced by addition of cell extracts (Ruch *et al.* 1974). The assays contained the following components: 100 mmol $l^{-1}$ glycerol or 1,3-PD; 2 mmol $l^{-1}$ $NAD^+$; 30 mmol $l^{-1}$ ammonium sulphate and 100 mmol $l^{-1}$ potassium carbonate buffer (pH 9·0). For the effect of the NADH/$NAD^+$ ratio, the concentration of $NAD^+$ used was 0·2 mmol $l^{-1}$.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activity was measured by the linear increase in $A_{340}$ produced by the addition of cell extracts (Ferdinand 1964). For the effect of the NADH/$NAD^+$ ratio, the concentration of $NAD^+$ was 0·1 mmol $l^{-1}$.

Acetate kinase (EC 2.7.2.1) and butyrate kinase (EC 2.7.2.7) activities, in the non-physiological direction, were determined by measuring the rate of acetyl- or butyryl-phosphate produced by the method of Lipman and Tuttle (1945) at 540 nm with acetyl-phosphate as standard. The assays contained 100 mmol $l^{-1}$ Tris-HCl buffer (pH 7·5); 0.4 mol $l^{-1}$ potassium acetate or 0·4 mol $l^{-1}$ potassium butyrate; 6 mmol $l^{-1}$ manganese sulphate and 10 mmol $l^{-1}$ ATP. After 5 min at 37 °C, 0·7 mol $l^{-1}$ hydroxylamin, pH 6·4 (freshly neutralized), was added. After 5 min at room temperature, 15% (TCA 4 mol $l^{-1}$ HCl and 5% $FeCl_3$ were added.

Levels of $NAD^+$ and NADH were measured after extraction of a culture broth sample, neutralization and filtration. Samples of 4 ml were taken quickly from the bioreactor with sterile syringes and immediately put into tubes containing the extractant, without separating the cells from the medium.

$NAD^+$ was extracted with HCl (NADH and NADPH were degraded) and NADH was extracted with KOH (degrading $NAD^+$ and $NADP^+$) as described by Wimpenny and Firth (1972). Before assays for NADH, this coenzyme was converted to $NAD^+$ with lactate dehydrogenase (EC 1.1.1.28) (Klingenberg 1965). $NAD^+$ was assayed with an NAD(H)-specific alcohol dehydrogenase (EC 1.1.1.1) (Klingenberg 1965; Vasconcelos *et al.* 1994) and the resulting

NADH was determined by fluorometry (Hitachi, Tokyo, Japan; model F-2000).

## RESULTS

### Effect of glucose on glycerol utilization

Results obtained on a glucose–glycerol batch culture in a molar ratio of 1, inoculated with cells grown on glycerol, are shown in Fig. 1. There was a significant lag phase in glycerol utilization during the first 5 h whereas glucose was initially metabolized. When glycerol was metabolized a decrease in glucose utilization was observed and then both substrates were used. Similar phenomena were observed with an inoculum grown on glucose (Fig. 2) and whatever the glucose–glycerol ratios (data not shown).

Table 1 compares the results observed on mixtures of both substrates with those obtained with glucose and glycerol alone.

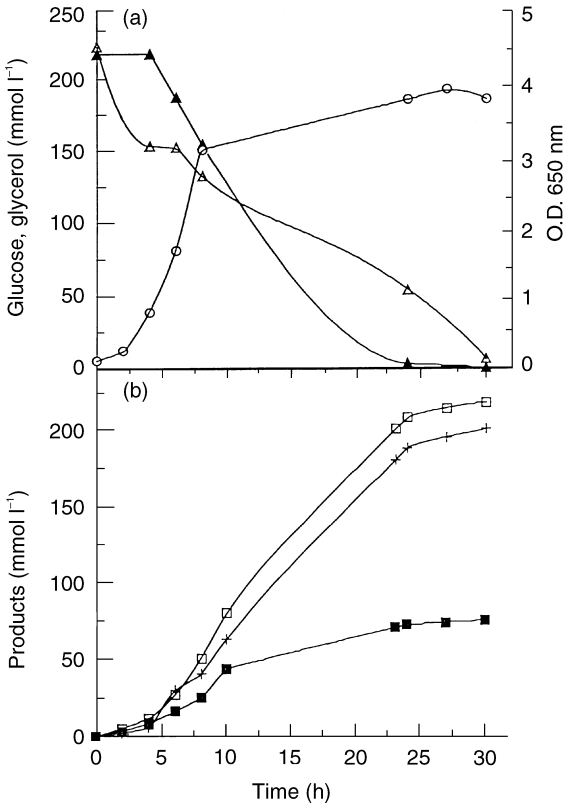


**Fig. 1** *Clostridium butyricum* DSM 5431 (a) growth and substrate utilization and (b) product formation in a culture fermenter with a mixture of 222 mmol $l^{-1}$ glucose and 218 mmol $l^{-1}$ glycerol. The batch culture was inoculated with cells grown on glycerol. ○, Growth; △, glucose; ▲, glycerol; □, acetic acid; ■, butyric acid; +, 1,3-propanediol

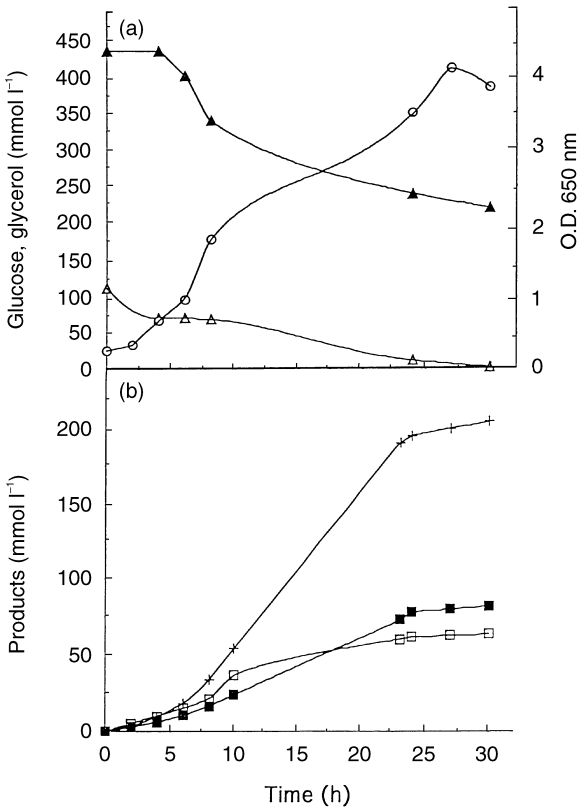


**Fig. 2** *Clostridium butyricum* DSM 5431 (a) growth and substrate utilization and (b) product formation in a culture fermenter with a mixture of 111 mmol $l^{-1}$ glucose and 436 mmol $l^{-1}$ glycerol. The batch culture was inoculated with cells grown on glucose. ○, Growth; △, glucose; ▲, glycerol; □, acetic acid; ■, butyric acid; +, 1,3-propanediol

During glycerol fermentation, acetate, butyrate and propanediol were excreted whereas only acetate and butyrate were excreted during glucose fermentation. When *Cl. butyricum* was cultured on a medium containing both substrates, and whatever the origin of the inoculum, the glycerol conversion to 1,3-PD increased sharply from 0·57 to 0·92–0·93 mol 1,3-PD produced $mol^{-1}$ glycerol used.

Table 2 shows the catabolic pathway flux calculated during 24 h of fermentation. A lower rate of glucose catabolism ($q_{glucose}$ = 4·02 mmol $h^{-1}$ $g^{-1}$) was measured compared with that of glycerol catabolism ($q_{glycerol}$ = 11·2 mmol $h^{-1}$ $g^{-1}$). Since the whole of the 1,3-PD was produced from the glycerol it is easy to calculate that, on a mixture of glucose–glycerol, the cells chiefly used the glycerol ($q_{glycerol}$ = 6·42 and 7·35 mmol $h^{-1}$ $g^{-1}$) to produce 1,3-PD ($q_{1,3\text{-PD}}$ = 5·90 and 6·90 mmol $h^{-1}$ $g^{-1}$) whereas glucose was used to produce acetate and butyrate.

**Table 1** Product formation by *Clostridium butyricum* DSM 5431 grown on glucose, glycerol or mixtures of glucose–glycerol

| Inoculum | Substrates (mmol $l^{-1}$) | Glycerol used (mmol $l^{-1}$) | Glucose used (mmol $l^{-1}$) | Products formed (mmol $l^{-1}$) | | | | Carbon recovery, (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | Acetate | Butyrate | 1,3-PD | $Y_{1,3\text{-PD}}$ | |
| Glycerol | Glycerol (218)* | 218 (11)† | — | 53·3 (3·7) | 20·5 (1·6) | 123·7 (11·1) | 0·57 | 100 |
| Glycerol | Glycerol–glucose (218–222) | 218 (12) | 188 (9) | 218·3 (15·2) | 75·0 (4·5) | 200 (14·2) | 0·92 | 95·6 |
| Glucose | Glucose (111) | — | 111 (6) | 63·3 (4·4) | 73·9 (5·8) | np | — | 95·1 |
| Glucose | Glucose–glycerol (111–436) | 218 (13) | 111 (8) | 68·3 (4·7) | 81·8 (4·9) | 204 (14·3) | 0·93 | 99·1 |

* Data in parentheses are substrate concentrations expressed in mmol $l^{-1}$.
† Data in parentheses are S.D. values of substrate and product concentrations from four determinations.
np, Not possible; 1,3-PD, 1,3-propanediol.
Carbon recovery values were calculated with theoretical $CO_2$ without including substrate consumptions for biomass formation which are small; glycerol and glucose conversion were determined as previously described by Biebl and Marten (1995).

**Table 2** Catabolic pathway fluxes on glucose, glycerol or mixtures of glucose–glycerol

| Inoculum | Substrates | Fermentation time (h) | $q_{glycerol}$ | $q_{glucose}$ | $q_{acetate}$ | $q_{butyrate}$ | $q_{1,3\text{-PD}}$ |
|---|---|---|---|---|---|---|---|
| Glycerol | Glycerol (218 mmol $l^{-1}$) | 24 | 11·20 | — | 2·74 | 1·05 | 6·35 |
| Glycerol | Glycerol–glucose (218–222 mmol $l^{-1}$) | 24 | 6·42 | 5·58 | 6·45 | 2·01 | 5·90 |
| Glucose | Glucose (111 mmol $l^{-1}$) | 24 | — | 4·02 | 2·29 | 2·76 | — |
| Glucose | Glucose–glycerol (111–436 mmol $l^{-1}$) | 24 | 7·35 | 3·76 | 2·31 | 2·77 | 6·90 |

Pathway flux values are expressed in mmol $h^{-1}$ $g^{-1}$.
1,3-PD, 1,3-propanediol.

### Levels of key metabolic enzyme activities from cell extracts of *Cl. butyricum* grown on glucose, glycerol or glucose–glycerol mixtures

No GDH or 1,3-PD dehydrogenase activity was detected in cell-free extracts of glucose-grown cells. Whatever the growth conditions, acetate kinase activities were higher than those of butyrate kinase and were detected in the same range of activity during the growth phases (Table 3). In contrast, in glucose–glycerol mixture-grown cells, with both types of inoculum, GDH and 1,3-PD dehydrogenase activities were low during the early exponential phase of growth and then reached the same activity range as for cells grown on glycerol (Table 3). From these data, it is clear that glucose has inhibitory effects on the induction of the enzymes implicated in glycerol catabolism during the early exponential phase and hence on the specific rates of glycerol uptake (Fig. 3). When the glycerol–glucose ratio was progressively changed from 0·98 to 3·89, the ratio $q_{glycerol}$–$q_{glucose}$ was between 0·24 and 0·30 (Fig. 3a). A shift occurred during the early decelerating growth phase with a ratio $q_{glycerol}$–$q_{glucose}$ of between 11·6 and 14·8 (Fig. 3b).

When the sugar mixture glycerol–glucose ratio was progressively changed from 0·48 to 1·94 and inoculated with cells grown on glycerol, formation of GDH and 1,3-PD dehydrogenase was repressed by the glucose concentration (Table 3) and hence, the specific rates of glycerol utilization decreased at the start of the fermentation (Fig. 4a). During the early decelerating growth phase, GDH and 1,3-PD dehydrogenase activities were similar to those found in extracts of cells grown on glycerol (Table 3) concomitant with higher specific rates of utilization of glycerol than glucose, whatever the glucose concentration added to the culture medium (Fig. 4b).

### Kinetic properties of glyceraldehyde-3-phosphate dehydrogenase and glycerol dehydrogenase

When glycerol is used as carbon source, glyceraldehyde-3-phosphate production is dependent on GDH but when

**Table 3** Enzymic activities from cell-free extracts of glucose, glycerol or mixtures of glucose–glycerol from batch cultures of *Clostridium butyricum* DSM 5431

| | Inoculum glycerol | | | | | | Inoculum glucose | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glycerol | | | Glycerol–glucose | | | Glucose | | | Glucose–glycerol | | |
| Enzyme | I | II | III | I | II | III | I | II | III | I | II | III |
| Acetate kinase | 8·3 (0·9) | 5·61 (0·6) | 6·42 (0·7) | 7·3 (0·8) | 5·84 (0·5) | 6·82 (0·7) | 3·4 (0·4) | 7·71 (0·6) | 9·92 (0·9) | 5·1 (0·6) | 5·63 (0·6) | 6·67 (0·7) |
| Butyrate kinase | 1·7 (0·2) | 2·81 (0·3) | 3·33 (0·3) | 2·6 (0·3) | 2·64 (0·2) | 3·62 (0·4) | 2·8 (0·3) | 3·11 (0·3) | 4·50 (0·5) | 1·9 (0·6) | 4·04 (0·4) | 5·42 (0·6) |
| Glycerol dehydrogenase | 0·31 (0·03) | 0·63 (0·05) | 0·96 (0·10) | 0·06 (0·01) | 0·68 (0·07) | 0·99 (0·11) | np | np | np | 0·08 (0·01) | 0·65 (0·07) | 1·48 (0·15) |
| 1,3-PD dehydrogenase | 0·42 (0·04) | 0·61 (0·06) | 1·25 (0·13) | 0·08 (0·01) | 0·65 (0·05) | 1·27 (0·12) | np | np | np | 0·04 (0·01) | 0·62 (0·05) | 1·38 (0·14) |

I, Early exponential growth phase; II, early decelerating growth phase; III, end of the growth phase; np, not possible; 1,3-PD, 1,3-propanediol.
Data in parentheses are S.D. values from four determinations. Substrate concentrations are given in Table 2.

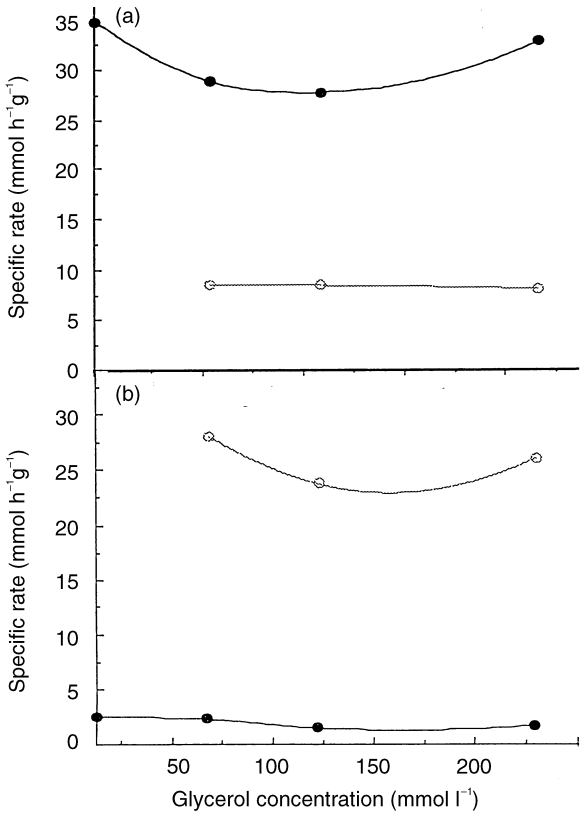


**Fig. 3** Influence of glycerol concentration on specific rates of glucose utilization by *Clostridium butyricum* DSM 5431 in (a) early exponential growth phase and (b) early decelerating growth phase. Concentration of glucose was 55·5 mmol $l^{-1}$ and glycerol from 54·3 to 218. Cultures were inoculated from a glucose-grown culture. ●, Glucose; ○, glycerol

glycerol and glucose are used simultaneously, the origin of the glyceraldehyde-3-phosphate can be glucose or glycerol.

On glycerol alone, GAPDH is linked to the GDH activity but on a mixture of substrates, glyceraldehyde-3-phosphate production can be concerned only by glucose catabolism and in this case the link between GDH and GAPDH disappears. This is why the properties of both enzymes were investigated in order to understand the observed changes in metabolism.

The apparent $K_m$ values for the reactions catalysed by the GAPDH and GDH were calculated from standard Lineweaver–Burk plots and are summarized in Table 4.

The affinities of the two dehydrogenases for $NAD^+$ varied considerably; the apparent $K_m$ for GDH was almost fourfold higher than that for GAPDH. The affinities for the second substrate were also different since the apparent $K_m$ for GDH was 16-fold higher for the glycerol than for the glyceraldehyde-3-phosphate in the case of GAPDH.

The effect of NADH on GAPDH and GDH activities was determined, since high intracellular levels of NADH were

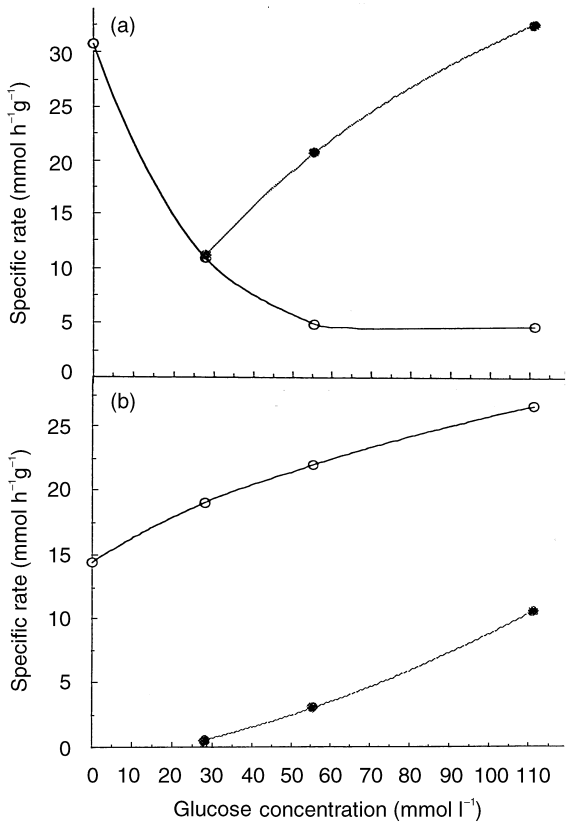


**Fig. 4** Influence of glucose concentration on specific rates of glycerol utilization by *Clostridium butyricum* DSM 5431 in (a) early exponential growth phase and (b) early decelerating growth phase. Concentration of glycerol was 108 mmol $l^{-1}$ and glucose from 27·8 to 111 mmol $l^{-1}$. Cultures were inoculated from a glycerol-grown culture. ●, Glucose; ○, glycerol

**Table 4** Determination of $K_m$ values for substrates and coenzymes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol dehydrogenase (GDH) from *Clostridium butyricum* DSM 5431

| Substrate or coenzyme tested | | Other reactant | $K_m$ (mmol $l^{-1}$)* |
|---|---|---|---|
| GAPDH | G3P | 1 mmol $l^{-1}$ $NAD^+$ | 1·65 (0·32) |
| | $NAD^+$ | 20 mmol $l^{-1}$ G3P | 0·082 (0·013) |
| GDH | Glycerol | 2 mmol $l^{-1}$ $NAD^+$ | 26·36 (5·59) |
| | $NAD^+$ | 100 mmol $l^{-1}$ glycerol | 0·322 (0·075) |

* $K_m$ values were calculated by non-linear regression to the Michaelis–Menten equation using the curve Fit feature of the program Grafit (Erithacus Software).
S.D. (of three determinations) is given in parentheses.

found with NADH–$NAD^+$ ratios between 0·7 and 3·2 (Table 5). The results (Fig. 5) show that, as the level of NADH was increased, the rate of $NAD^+$ reduction by GAPDH was less inhibited than that of GDH.

## DISCUSSION

Growth of *Cl. butyricum* on glycerol is preceded by the induction of four enzymes: GDH, DHA kinase, glycerol dehydratase and 1,3-PD dehydrogenase (Dabrock *et al.* 1992; Abbad-Andaloussi *et al.* 1996). In this study, it is shown that the induced synthesis of these enzymes is repressed by the inclusion of glucose in the growth medium chiefly at the start of fermentation; the levels of 1,3-PD dehydrogenase and GDH were then similar to those found in extracts of cells grown on glycerol. Therefore, glucose was used alone during early exponential growth followed by simultaneous glucose–glycerol consumption. With glucose–glycerol mixtures there was a sharp increase in the conversion of glycerol into 1,3-PD. On glycerol alone, 57% of the glycerol was diverted through the 1,3-PD pathway and 43% through the DHA pathway, whereas the theoretical maximum propanediol yield was 72% with the assumption that no hydrogen and no butyric acid were produced (Zeng *et al.* 1993). Saint-Amans and Soucaille (1995) studied the influence of glucose as a co-substrate in glycerol fermentation to 1,3-PD by *Cl. butyricum* VPI 3266 in carbon-limited chemostat cultures. These authors reported increased propanediol yields based on glycerol consumption but the maximum propanediol yield obtained was 0·60 mol propanediol $mol^{-1}$ glycerol used. Contrary to these results, Biebl and Marten (1995) showed that, for the mixed-substrate culture, 90% of the glycerol was converted to 1,3-PD and 10% was used for acids, enhancement of the 1,3-PD yield by glucose being obtained only when the preculture was grown on glycerol. Our results are in line with those of Biebl and Marten (1995) since 92–93% of the glycerol was converted through the 1,3-PD pathway, but they also indicate that the enhancement of the 1,3-PD yield was obtained whatever the nature of the carbon source of the preculture.

However, we found that the conversion yield of glycerol into 1,3-PD was rather constant between 0·61 and 0·65 mol propanediol $mol^{-1}$ glycerol used, when *Cl. butyricum* was grown in continuous culture or in fed-batch culture (Abbad-Andaloussi *et al.* 1995, 1996). The relatively constant value of the conversion yield was due to the presence of regulatory mechanisms that precisely partition the carbon flow to avoid intracellular concentration of 3-HPA, a very toxic compound. Nevertheless, in the presence of glucose, the conversion of glycerol into 1,3-PD changed markedly in comparison with pure glycerol fermentations. When both substrates are consumed, glucose catabolism is used by the cells to produce energy and NADH, whereas glycerol is used chiefly to pro-

**Table 5** Nucleotide levels of *Clostridium butyricum* DSM 5431 grown on glucose, glycerol and mixtures of glucose–glycerol

| | | Nucleotide conc. [$\mu$mol (g dry cell wt)$^{-1}$] | | | | | |
|---|---|---|---|---|---|---|---|
| | | $NAD^+$ | | NADH | | $NADH/NAD^+$ | |
| Inoculum | Substrates | I | II | I | II | I | II |
| Glycerol | Glycerol (218)* | 5·3 (0·4)† | 4·8 (0·3) | 16·5 (1·9) | 15·6 (0·6) | 3·1 | 3·2 |
| Glycerol | Glycerol–glucose (218–222) | 15·2 (1·4) | 16·4 (1·7) | 27·3 (2·9) | 13·8 (1·5) | 1·8 | 0·8 |
| Glucose | Glucose (111) | 7·9 (0·7) | 4·7 (0·3) | 18·1 (1·9) | 10·2 (0·9) | 2·3 | 2·1 |
| Glucose | Glucose–glycerol (111–436) | 15·8 (1·7) | 16·6 (1·8) | 26·6 (2·8) | 12·5 (1·5) | 1·7 | 0·7 |

I, Exponential phase of the growth; II, end of the growth.
* Data in parentheses are substrate concentrations expressed in mmol $l^{-1}$.
† Data in parentheses are S.D. values of substrate and product concentrations from four determinations.

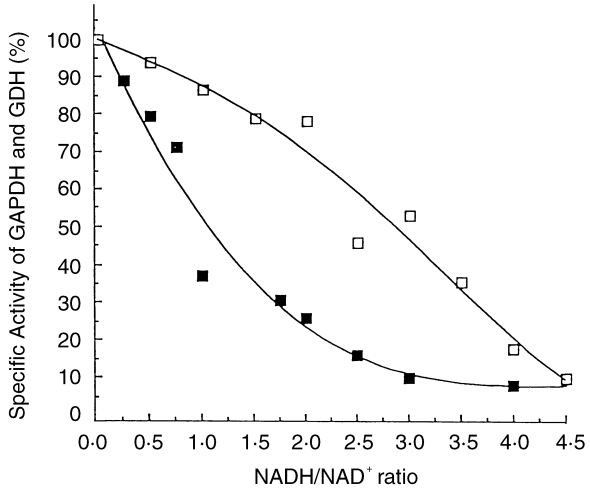


**Fig. 5** Influence of the $NADH/NAD^+$ ratio on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol dehydrogenase (GDH) in *Clostridium butyricum*. □, GAPDH; ■, GDH

duce 1,3-PD, resulting in oxidation of NADH and hence an enhanced yield of 1,3-PD.

From a metabolic point of view we can explain these data by the fact that the apparent $K_m$ values for GDH were markedly higher than those for GAPDH and the fact that GDH was more inhibited by NADH than was GAPDH. These kinetic properties are consistent with a weak GDH activity and hence a shift of the glycerol carbon flow towards 1,3-PD formation when glucose and glycerol are used simultaneously.

## ACKNOWLEDGEMENTS

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## REFERENCES

Abbad-Andaloussi, S., Manginot-Dürr, C., Amine, J., Petitdemange, E. and Petitdemange, H. (1995) Isolation and characterization of *Clostridium butyricum* DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. *Applied and Environmental Microbiology* **61**, 4413–4417.

Abbad-Andaloussi, S., Dürr, C., Raval, G. and Petitdemange, H. (1996) Carbon and electron flow in *Clostridium butyricum* grown in chemostat culture on glycerol and on glucose. *Microbiology* **142**, 1149–1158.

Biebl, H. and Pfenning, N. (1982) Isolation of members of the family Rhodospirillaceae. In *The Procaryotes* ed. Starr, M.P., Stolp, H., Trüper, H.G., Balows, A. and Schlegel, H.G. pp. 267–273. Berlin: Springer.

Biebl, H., Marten, S., Hippe, H. and Deckwer, W.D. (1992) Glycerol conversion to 1,3-propanediol by newly isolated clostridia. *Applied Microbiology and Biotechnology* **36**, 592–597.

Biebl, H. and Marten, S. (1995) Fermentation of glycerol to 1,3-propanediol: use of cosubstrates. *Applied Microbiology and Biotechnology* **44**, 15–19.

Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Dabrock, B., Bahl, H. and Gottschalk, G. (1992) Parameters affecting solvent production by *Clostridium pasteurianum*. *Applied Microbiology and Biotechnology* **58**, 1233–1239.

Ferdinand, W. (1964) The isolation and specific activity of rabbit-muscle glyceraldehyde phosphate dehydrogenase. *Biochemical Journal* **92**, 578–585.

Jungermann, K., Thauer, R.K., Leimenstoll, G. and Decker, K. (1973) Function of reduced pyridine nucleotide-ferredoxin oxidoreductases in saccharolytic clostridia. *Biochimica et Biophysica Acta* **305**, 268–280.

Klingenberg, M. (1965) Nicotinamide-adenine dinucleotides (NAD, NADP, NADH, NADPH). Spectrophotometric and fluorometric methods. In *Methods of Enzymatic Analysis*, Vol. 4, 2nd edn. ed. Bergmeyer, H.U. pp. 2045–2059. New York: Academic Press.

Lipmann, F. and Tuttle, L.C. (1945) A specific micro-method for determination of acyl-phosphates. *Journal of Biological Chemistry* **159**, 21–28.

Ruch, F.E., Lengeler, J. and Lin, E.C. (1974) Regulation of glycerol catabolism in *Klebsiella aerogenes*. *Journal of Bacteriology* **119**, 50–56.

Saint-Amans, S. and Soucaille, P. (1995) Carbon and electron flow in *Clostridium butyricum* grown in chemostat culture on glucose-glycerol mixtures. *Biotechnology Letters* **17**, 211–216.

Vasconcelos, I., Girbal, L. and Soucaille, P. (1994) Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixture of glucose and glycerol. *Journal of Bacteriology* **176**, 1443–1450.

Wimpenny, J.W.T. and Firth, A. (1972) Levels of nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide in facultative bacteria and the effect of oxygen. *Journal of Bacteriology* **111**, 24–32.

Zeng, A.P., Biebl, H., Schlieker, H. and Deckwer, W.D. (1993) Pathway analysis of glycerol fermentation by *Klebsiella pneumoniae*: regulation of reducing equivalent balance and product formation. *Enzyme Microbiology and Technology* **15**, 770–779.