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# Glycerol fermentation of 1,3-propanediol by *Clostridium butyricum*. Measurement of product inhibition by use of a pH-auxostat

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**Summary.** The fermentation of glycerol to 1,3-propanediol, acetate, and butyrate by *Clostridium butyricum* was studied with respect to growth inhibition by the accumulating products. The clostridia were grown in a pH-auxostat culture at low cell density and product concentration and near maximum growth rate. The products were then added individually to the medium in increasing concentrations and the resulting depression of growth rate was used as a quantitative estimate of product inhibition. Under these conditions growth was totally inhibited at concentrations of 60 g/l for 1,3-propanediol, 27 g/l for acetic acid and 19 g/l for butyric acid at pH 6.5. Appreciable inhibition by glycerol was found only above a concentration of 80 g/l. In a pH-auxostat without added products but with high cell density as well as in batch cultures the product proportions were different. The 1,3-propanediol concentration may approach the value of complete inhibition while the concentrations of acetic and butyric acids remained below these values by at least one order of magnitude. It was therefore concluded that 1,3-propanediol is the first range inhibitor in this fermentation.

## Introduction

Fermentation of glycerol by clostridia to 1,3-propanediol, butyrate and acetate has been described by Nakas et al. (1983) and Forsberg (1987). Although the main product 1,3-propanediol might be of interest for polymer synthesis no further work has initiated development of this process. We have recently isolated a strain of *Clostridium butyricum* that is able to ferment glycerol up to a concentration of 100 g/l to about 55 g/l of propanediol together with acetic and butyric acids (unpublished data). As in other fermentations the limited substrate conversion is a consequence of product inhibition. It would therefore be of great interest for improving the process to find out to which extent each of the three products contributes to this inhibition.

In several mixed fermentations, inhibition by the individual products has been determined by measuring the growth rate in batch cultures to which the substance in question was added in increasing concentration (Costa and Moreira 1983; Fond et al. 1985; de Mas et al. 1987). For the acetone-butanol fermentation it was found that butanol itself is the main inhibitor, whereas in the butanediol fermentation of *Enterobacter-Klebsiella* the main product is tolerated in relatively high concentrations, and growth inhibition is mainly caused by acetic acid in microaerobic culture or by ethanol, when the conditions are anaerobic (Zeng et al. 1990; Zeng and Deckwer 1991).

To quantify the effect of stimulating or inhibiting agents, continuous cultures have repeatedly been used. The pH-auxostat as a nutrient-sufficient continuous culture (Martin and Hempfling 1976; Fraleigh et al. 1989) allows measurement of the influence of added substances directly by a change in the dilution rate (=growth rate) in a similar way to the above simpler method, but with better accuracy and reproducibility (Larsson and Enfors 1990). In the present paper the action of the three products of clostridial glycerol fermentation is analysed by adding them separately to a culture that grows near its maximal specific rate. The results may help to improve the culture techniques in order to achieve higher concentrations and better productivity of 1,3-propanediol.

## Materials and methods

*Organism and medium. C. butyricum* strain DSM 5431 (=SH1; European patent applied for, no. EP 0361082A2) was used. The strain was maintained on anaerobically incubated milk agar plates (milk and 4% agar mixed in a proportion of 1:1) to obtain spores. 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 medium contained per litre deionized water if not otherwise stated: glycerol, 20 g; $K_2HPO_4$, 3.4 g; $KH_2PO_4$, 1.3; $(NH_4)_2SO_4$, 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; $CaCO_3$, 2 g; yeast extract, 1 g; trace element solution SL 7 (Biebl and Pfennig 1981), 2 ml. If used for pH-controlled fermen-

tations the phosphate concentration was reduced to 1 g $K_2HPO_4$ and 0.5 g $KH_2PO_4$, and $CaCO_3$ was omitted.

*Continuous culture.* A pH-auxostat version with a separate medium and alkali supply was used (Oltmann et al. 1978). The culture vessel was a 1-l double walled fermentor (BCC, Göttingen, FRG) with a working volume of 300 ml. The pH-controller was a no longer manufactured model (Jungkeit, Göttingen, FRG) that allowed adjustment of pulse and interval to a ratio of 1:1 (5 s each). Alkali (0.446 M KOH) and medium were pumped synchronously into the fermentor according to the signal of the pH controller. The KOH pump rate was varied between 28 and 280 ml/h while the medium pump operated at a constant rate of 840 ml/h.

The culture volume was controlled by weight using scales and a weight control unit from Sartorius, Göttingen, FRG. The alkali flow and the effluent were measured from weight decrease or increase, respectively, and were continuously recorded.

The growth temperature was kept at 32° C using an external thermostat. If not otherwise stated the pH was controlled at 6.5.

*Analytical methods.* Glycerol was determined enzymatically via glycerol kinase and L-lactate dehydrogenase using the test kit and instructions of Boehringer, Mannheim, FRG. The fermentation products were analysed gas chromatographically on a 1-m Chromosorb 101 column using a stepwise temperature programme from 150 to 200° C (7°/min initially, 14° C/min after 1.5 min), nitrogen as carrier gas and *n*-butanol as the internal standard. The cell density was measured at 650 nm in a 1-cm cuvette.

*Theoretical concept.* The relationships between the growth parameters in a pH-auxostat have been repeatedly formulated (Martin and Hempfling 1976; Büttner et al. 1986; Fraleigh et al. 1990). While the growth rate under steady-state conditions is normally near its maximum, the cell density can be determined by the buffering capacity of the medium. Provided the culture fluid is acidified during growth the cell density can be expressed as:

$$\bar{x} = Y_{H^+} \cdot BC_R$$

according to Büttner et al. (1986), where $Y_{H^+}$ = biomass formed per of $H^+$ ions produced and $BC_R$ = buffering capacity = amount of $H^+$ ions to bring the pH of 1 l medium to the pH set-point of the culture.

In the experimental set-up, medium and alkali are fed in separate lines, and the medium pH is equal to the pH set-point of the culture. Accordingly $BC_R$ corresponds to the amount of alkali ($A_0$) being fed to the culture relative to the medium, and $Y_{H^+}$ is equivalent to cell mass formed per alkali added ($Y_{X/A}$):

$$\bar{x} = Y_{X/A} \cdot A_0.$$

Product concentration ($\bar{P}$) and substrate consumption ($\bar{S}$) depend equally on $A_0$:

$$\bar{P} = Y_{P/A} \cdot A_0$$
$$\bar{S} = Y_{S/A} \cdot A_0.$$

$A_0$ (called alkali supply in the following) is calculated from the concentration of the alkali solution ($A_R$) and the flow rates of the alkali solution ($F_A$) and the medium ($F_M$):

$$A_0 = A_R \cdot \frac{F_A}{F_M + F_A}.$$

In this investigation the alkali flow was varied at a constant alkali concentration and medium flow. A relatively low alkali concentration had to be used to prevent detrimental effects on the *Clostridium* culture.

When the fermentation products accumulate, e.g. by adjusting higher alkali flow rates, growth falls below the maximum rate due to the combined action of the three inhibiting products. The effect of an individual product can be measured if it is added to a steady state pH-auxostat culture of low cell density and correspondingly low concentrations of products formed by the culture.

## Results

### *Influence of the products formed during the fermentation on growth*

To assess the basic parameters of the clostridial glycerol fermentation in a pH-auxostat, increasing product concentrations and cell densities were adjusted by increasing the alkali flow rate at constant medium flow. Figure 1 shows the resulting dilution rates together with the cell densities, product concentrations and glycerol consumption at a pH value of 7.0. Growth at the maximum rate of 0.68 $h^{-1}$ was found only up to the fairly low product concentration of 5.5 g/l of 1,3-propanediol, 1.0 g/l of acetic acid, and 0.8 g/l of butyric acid. Beyond these concentrations growth was increasingly impaired.

While the propanediol concentration increased almost linearly with KOH supply, the two acids were formed at a different rate when the product concentration increased, i.e. butyric acid formation was favoured in relation to acetic acid. This behaviour was observed under all conditions of inhibition and is discussed below.

The optical density increased up to a point where the growth rate became half maximal, but levelled off at lower growth rates while the products still increased. Cells became elongated to filamentous under conditions of high product concentration.

### *Influence of the pH value*

When the pH is lowered in a pH-auxostat culture the alkali requirement reduces if the ratio alkali flow/me-

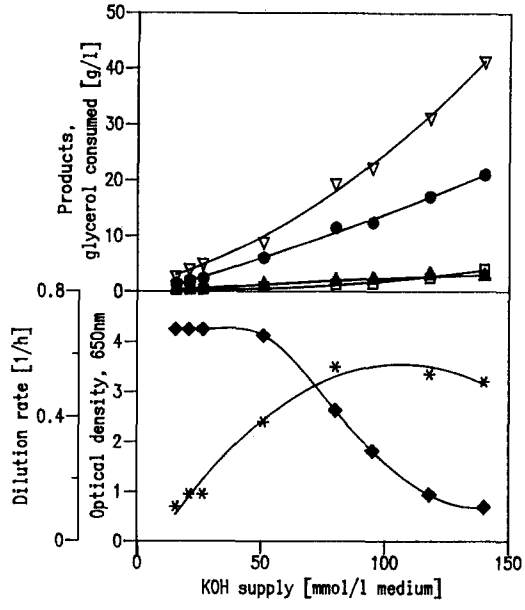


**Fig. 1.** Growth of *Clostridium butyricum* DSM 5431 in a pH-auxostat culture at increasing product concentrations adjusted by variation of the alkali supply at pH 7.0: ▽, glycerol consumed; ●, 1,3-propanediol; ▲, acetic acid; □, butyric acid; ◆, dilution rate; *, optical density

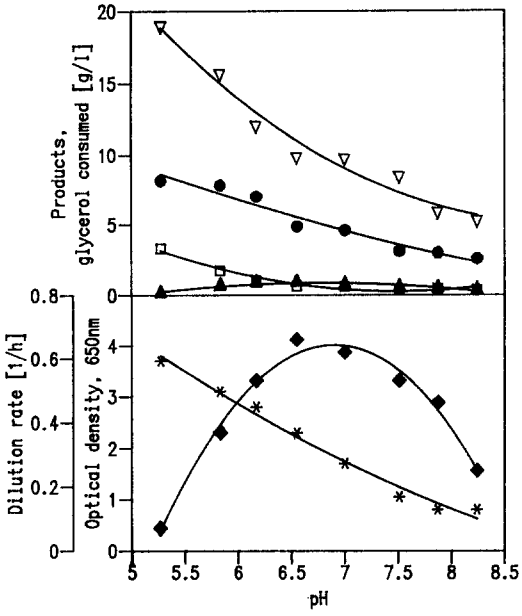


**Fig. 2.** pH-Auxostat culture at varied pH values and constant KOH supply (33 mmol/l medium): symbols as in Fig. 1

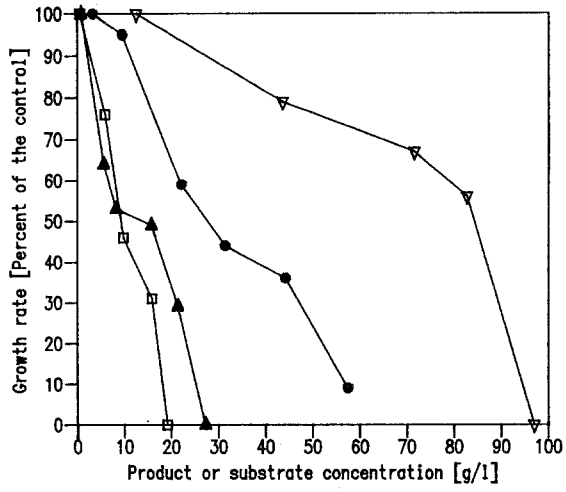


**Fig. 3.** Dependency of the dilution rate on the concentration of individually added products or of the substrate. The concentrations indicated are those that result under steady-state conditions: KOH-supply, 32–34 mmol/l; pH 6.5. Symbols as in Fig. 1

dium flow is fixed, and therefore cell density and product concentration increase. Such an experiment – varied pH at constant KOH supply – is shown in Fig. 2. The dilution rate was highest at about pH 6.7. The decrease above pH 7.0 appears to be a true pH effect, while the decline towards lower pH is obviously due to product inhibition. When the cell density was kept constant at different pH values by raising the KOH supply according to the above experiment (Fig. 2) the dilution rate remained almost at the same level from pH 7.0 down to 5.8 (not shown).

### *Influence of individually added products and influence of the substrate*

Experiments with addition of products were carried out at a pH of 6.5 and a KOH supply of 32–34 mmol/l medium, resulting in glycerol consumption of about 7 g/l, propanediol formation of 3.8 g/l, and a cell concentration of 0.63 g/l, before any of the substances were added. Under these conditions the dilution rate was still near its maximum and varied between 0.68 and 0.80/h from experiment to experiment. Figure 3 shows the influence of acetate, butyrate and 1,3-propanediol on the dilution rate when added in increasing concentration. Also shown is the action of the substrate, glycerol. Obviously 1,3-propanediol was the least toxic product, being tolerated up to a concentration of about 60 g/l. The acetic and butyric acids caused stronger inhibition; 27 and 19 g/l were sufficient for total growth inhibition at pH 6.5, respectively. Glycerol exerted only slight growth impairment up to about 80 g/l in the culture. However, at 97 g/l in the culture corresponding to 122 g/l in the feed, growth and medium request ceased entirely.

External addition of products and substrate also influenced product proportions and substrate consumption (Table 1). Generally speaking, the 1,3-propanediol concentration increased somewhat on product or sub-

**Table 1.** Concentrations of products formed and glycerol consumed at maximum concentration of added product or substrate tolerated

| Added substance | (g/l) | Optical density at 650 nm | Glycerol consumed (g/l) | 1,3-Propanediol formed (g/l) | Butyric acid formed (g/l) | Acetic acid formed (g/l) |
|---|---|---|---|---|---|---|
| Butyric acid | 0.0 | 1.7 | 7.6 | 3.8 | 0.5 | 0.7 |
| | 14.9 | 1.7 | 9.7 | 5.0 | n.d. | 0.2 |
| Acetic acid | 0.0 | 2.1 | 8.4 | 4.7 | 0.6 | 0.8 |
| | 20.6 | 1.7 | 8.6 | 3.1 | 1.2 | n.d. |
| 1,3-Propanediol | 0.0 | 1.8 | 6.3 | 3.2 | 0.5 | 0.7 |
| | 54.2 | 1.3 | 10.0 | 4.3 | 1.5 | 0.2 |
| Glycerol | 20.5 | 1.9 | 7.4 | 3.8 | 0.5 | 0.7 |
| | 103.3 | 1.6 | 19.2 | 4.9 | 1.4 | 0.3 |

n.d. = not determined

strate addition, while the cell density decreased. Glycerol consumption increased, in particular when glycerol itself was in great excess. Of particular interest is the proportion of butyrate to acetate, which is demonstrated in Fig. 4 for added 1,3-propanediol and glycerol as a percentage of the glycerol necessary for formation of the acids. It appears that under substrate-sufficient, non-inhibitory conditions the proportion of glycerol for butyrate to glycerol for acetate is about 1:1 (butyrate:acetate = 1:2), whereas under product or substrate stress more butyrate was regularly formed and less acetate. A similar, less pronounced shift occurred when the products accumulated by formation (Fig. 1) and at high pH-values (Fig. 2).

In Table 2 the product concentrations for half maximal and, if measured, for complete growth inhibition are listed for cultures with added and with self-formed products. For the two acids the concentration of the undissociated forms, which have been found to be responsible for inhibition effects in fermentations (Fond et al. 1985; Zeng et al. 1990), was calculated. The figures show that the acid concentrations encountered in uninfluenced cultures are about times 20 lower than those that cause inhibition. However, the concentrations of added and formed 1,3-propanediol at the half maximal growth rate differed only by a factor of 2. This implies that product inhibition in the clostridial glycerol fermentation is much more an effect of 1,3-propanediol than of butyric and acetic acids which, although more toxic to the organisms, occur in concentrations too low to affect the cells appreciably.

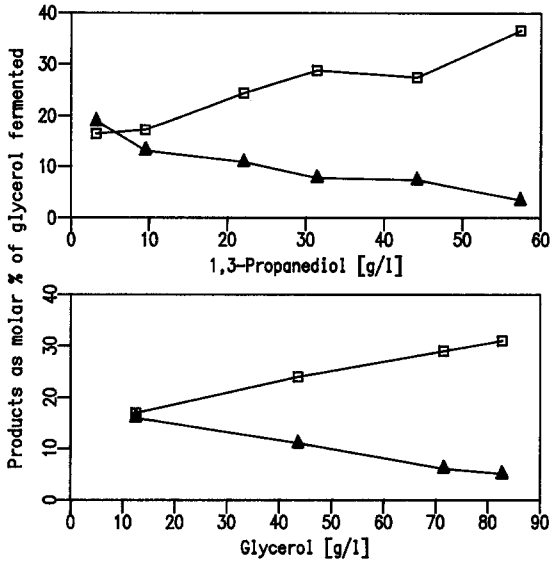


**Fig. 4.** Acetate (▲) and butyrate (□) concentrations relative to fermented glycerol under product and substrate stress

## Discussion

With respect to product composition and chemical properties of the products, 1,3-propanediol fermentation can be compared to 2,3-butanediol fermentation in the Enterobacteriaceae. Growth inhibition by the individual products in a butanediol-producing strain of *Klebsiella oxytoca* has been measured by Fond et al. (1985) in batch cultures. They determined a critical concentration of 105 g/l for 2,3-butanediol and of only 0.45 g/l for undissociated acetic acid. Thus, 2,3-butanediol is markedly less inhibitory than 1,3-propanediol, which gave a critical concentration of 60 g/l with *C. butyricum*, while the corresponding values for acetic acid are about the same for both organisms (cf. Table 2). Zeng et al. (1990) and Zeng and Deckwer (1991) investigated glucose fermentation by *Enterobacter aerogenes* in a substrate-sufficient chemostat culture and showed that the culture was strongly limited by acetate when grown microaerobically or by ethanol under anaerobic conditions, but not by butanediol. The determinative role of 1,3-propanediol in the clostridial glycerol fermentation may be attributed to the higher sensitivity of the cells to their main product, but also to the higher culture pH that is necessary for optimal solvent production.

An aspect specific to the fermentation investigated is the ability of the clostridia to vary the proportion of butyrate to acetate according to the environmental conditions. When glycerol was in excess and the product concentration low to medium, this proportion appeared to be 1:2, whereas under product or pH stress butyric acid increased while acetic acid decreased. Recently it was shown in glycerol-limited chemostat cultures with the same strain (Günzel 1991) that the acetate-butyrate proportion is also strictly dependent on the growth rate. At slow growth predominantly butyrate and at fast growth predominantly acetate formation was found.

**Table 2.** Inhibitory product concentrations in cultures with and without added product (g/l)

| pH-Auxostat culture | pH | Percentage of $\mu_{max}$ | 1,3-Propanediol | Acetic acid | | Butyric acid | |
|---|---|---|---|---|---|---|---|
| | | | | Total | Undissociated | Total | Undissociated |
| Without added products | 7.0 | 50 | 12 | 2.0 | 0.0115 | 1.3 | 0.0084 |
| With added products | 6.5 | 50 | 27 | 14 | 0.26 | 9 | 0.18 |
| | | 0 | 60 | 27 | 0.49 | 19 | 0.39 |

Both the auxostat and the chemostat observations point to an increased energy requirement when butyrate is excreted instead of acetate, as butyrate formation yields more ATP per mole of glycerol. As glycerol conversion to butyrate provides less reducing equivalents than conversion to acetate, the molar proportion of 1,3-propanediol is distinctly lower under these conditions. Thus, high product concentrations always mean an increased proportion of butyrate and accordingly a certain loss of the 1,3-propanediol yield which, however, does not exceed 10% of the optimum yield.

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