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Fermentation of glycerol by *Clostridium pasteurianum* — batch and continuous culture studies

H Biebl

German Research Center for Biotechnology, Brunswick, Germany

The fermentation of glycerol by *Clostridium pasteurianum* was studied with respect to product formation as influenced by the culture conditions. In the majority of batch cultures, butanol was the main fermentation product, but a varying fraction of glycerol was also converted to 1,3-propanediol, butyric and acetic acids and ethanol. More than 60 g/l glycerol was utilized, and up to 17 g/l butanol was produced. Fed-batch cultures did not offer an advantage. When molecular nitrogen was used as a nitrogen source, the fermentation time was prolonged by a factor of 1.5. Fermentations at constant pH values between 4.5 and 7.5 did not reveal significant differences in product formation except for an increase in the ethanol content starting at pH 6.5. Chemostat cultures also yielded predominantly *n*-butanol, but in some fermentations, the 1,3-propanediol fraction was relatively high. The pH auxostat cultures, which were operated at a glycerol excess, contained 1,3-propanediol as the main product. As a whole, the fermentations were characterized by a certain variability in product formation under seemingly equal or slightly varied conditions. It appears that the regulation of the numerous fermentation pathways occurring in this organism is not very strict. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 18–26.

Keywords: glycerol fermentation; *Clostridium pasteurianum*; *n*-butanol; 1,3-propanediol; chemostat; pH auxostat

Introduction

Fermentation of glycerol by *Clostridium pasteurianum* was first described in 1983 by Nakas *et al* [10] in an attempt to obtain a marketable product from photosynthetically formed glycerol by some halophilic algae. The main product of this fermentation type was *n*-butanol, but 1,3-propanediol was also produced in addition to ethanol and acetic acid. Heyndrickx *et al* [6] confirmed this product pattern in continuous cultures and showed that hydrogen is evolved corresponding to the acetyl-CoA formed. Dabrock *et al* [5] found markedly higher amounts of 1,3-propanediol and less butanol in batch fermentations of *C. pasteurianum*. Limitation by iron resulted in a shift from butanol to 1,3-propanediol. Recently, the genes for the two key enzymes of 1,3-propanediol formation from glycerol were cloned and expressed in *Escherichia coli* and found to be structurally closely related to the corresponding genes of *Klebsiella* and *Citrobacter* [8,9].

In contrast to *C. butyricum*, which produces 1,3-propanediol together with butyric and acetic acids, *C. pasteurianum*, in addition, is able to convert glycerol to butanol and ethanol. In Figure 1, the competing fermentation pathways are outlined. The environmental factors that lead these anaerobes to use one or the other metabolic route are not sufficiently known. In the present paper, an attempt is made to learn more about this fermentation by growing batch and continuous cultures under various conditions including pH, dilution rate and substrate concentration. The data obtained for butanol fermentation from glycerol are compared to the butanol–acetone fermentation of *C. acetobutylicum*.

Correspondence: Dr H Biebl, GBF, Mascheroder Weg 1, Braunschweig D-38124, Germany
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Materials and methods

Strains utilized

C. pasteurianum DSM 525 (type strain, identical with ATCC 6013) was used throughout. It was maintained as spores in dry sand.

Medium and fermentation conditions

The cultures were grown in a 1-l bioreactor (BCC, Göttingen, Germany) using a working volume of 500 ml for batch cultures and of 300 ml for continuous cultures. The temperature was controlled at 35°C, the pH at 6.0 or as indicated by 3.4 M KOH. Pure nitrogen was used as sparging gas at a rate of 0.1 vvm. For continuous cultures, the volume was maintained by a weight control device of Sartorius (Göttingen, Germany). Steady state parameters were assessed after five volume changes or more. Theory and operation of the pH auxostat have been previously described [1]. KOH solution and medium were pumped synchronously into the bioreactor according to the signal of the pH controller. The concentration of KOH (0.446 M) and medium flow rate (840 ml/h) were kept constant, while the KOH flow rate was varied. Precultures were grown in 100 ml septum bottles filled with 50 ml medium prepared anaerobically, the gas space being pure nitrogen [1]. They were used to inoculate the fermenter after 24 h of incubation at 30°C.

The following medium was used for bioreactor fermentations (amounts per liter of deionized water): KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4$, 5 mg, trace element solution SL 7 [3], 2 ml; glycerol, as indicated. The batch cultures contained 1 g yeast extract/l, if not otherwise stated; for the continuous cultures, yeast extract was replaced by biotin (25 μg/l). $(NH_4)_2SO_4$ was increased to 5 g/l in batch cultures with a

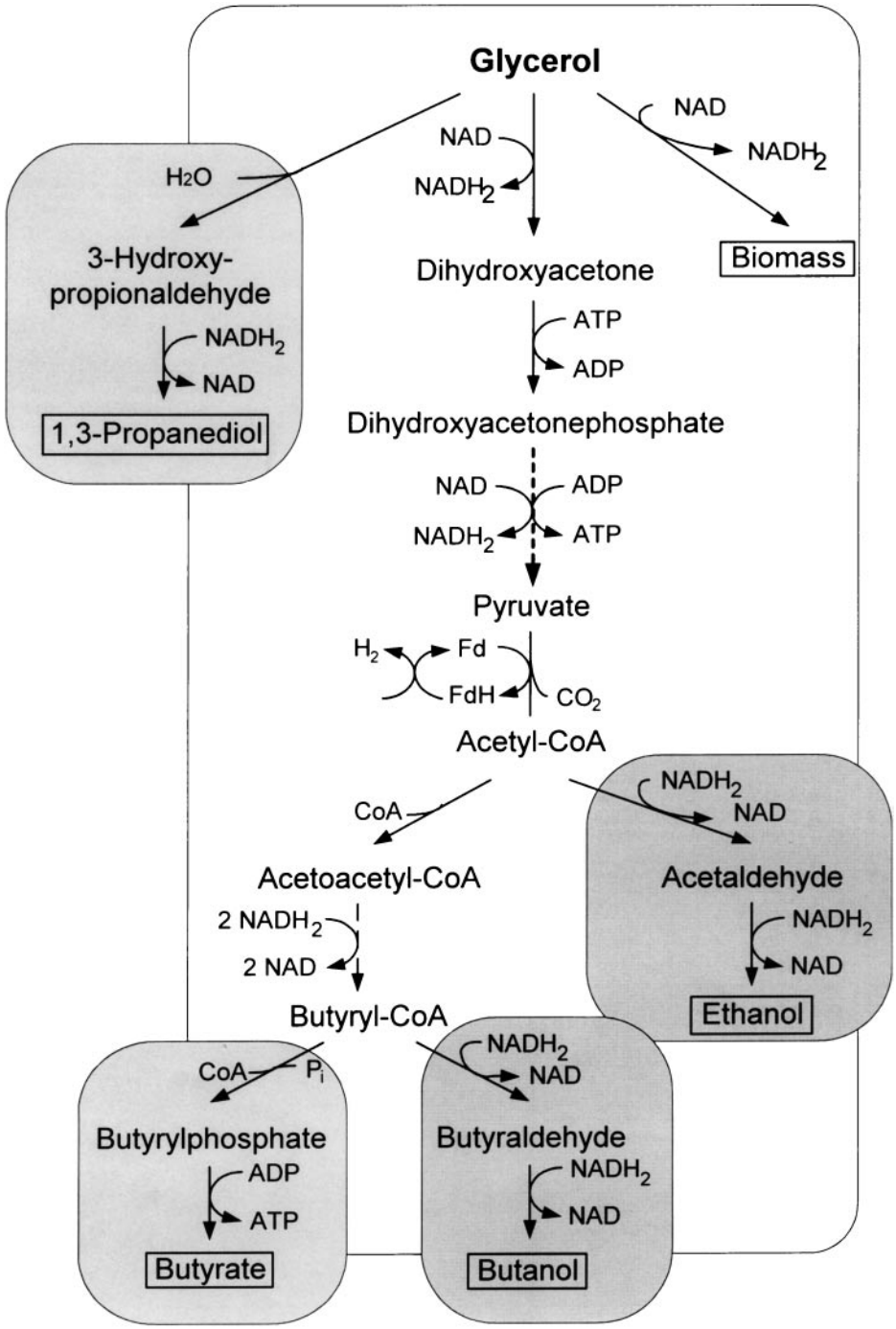

Figure 1 Metabolic pathways in the glycerol fermentation of *C. pasteurianum*. Light grey boxes: specific reactions of 1,3-propanediol/acids formation (acetate omitted); grey boxes: butanol or ethanol formation.

glycerol concentration higher than 50 g/l. Preculture bottles contained 2 g $CaCO_2$/l.

Analytical methods

Glycerol was determined enzymatically according to the instructions of the test kit manufacturer (Boehringer Mannheim, Mannheim, Germany). Fermentation products were measured gas chromatographically on Chromosorb 101 with nitrogen as carrier gas [1]. Cell dry weight was measured in preweighed 20 ml stainless steel centrifuge tubes; two 10 ml portions of culture were centrifuged, washed and dried for 24 h at 80°C.

Calculations

Recovery of products is given as molar ratio of the sum of products to the substrate (glycerol) consumed, whereby butanol and butyrate were multiplied by two, as 2 mol of glycerol are required for their formation.

Results

Product formation in batch cultures

Influence of pH: The role of pH, which is highly significant in many clostridial fermentations, was investigated in batch cultures controlled at values between 4.5 and 7.5 using glycerol at a concentration of 50 g/l. Table 1 shows the results. With the exception of pH 4.5, at which only part of the substrate was used after prolonged fermentation time, the cultures grew more or less equally well over the whole pH range with a slight preference for the weakly acidic range. The maximum growth rate was 0.37 h^{-1} at the substrate concentration used. The fermentation time was around 22 h.

In contrast, the variations in product formation were considerable, but a greater part of the glycerol was converted to butanol than to 1,3-propanediol, if it is considered that 2 mol of glycerol are consumed for 1 mol of butanol. A correlation with the pH applied was not found. Only ethanol formation showed a clear tendency: It increased from pH 6 to 7.5 and required up to 25% of the glycerol.

Figure 2 shows the course of a fermentation in which predominantly butanol is formed (pH 6.0). It can be seen that 1,3-propanediol and butyrate are produced from the beginning, whereas butanol formation starts with a delay of several hours. This feature reminds one of the sequence of acid and solvent formation in the acetone–butanol fermentation.

Influence of the glycerol concentration and fermentation in fed-batch culture: Fermentations controlled at pH 6 with different initial concentrations of glycerol showed that, due to product inhibition, the substrate was used only to a concentration greater than 60 g/l (Table 2). At higher substrate concentrations, conversion was slower, and glycerol remained in the culture. Again, the butanol/1,3-propanediol ratio varied considerably without noticeable relation to the applied glycerol concentration. As also shown in Table 2, fermentations starting with 50 g/l of glycerol followed by periodic glycerol addition did not lead to better substrate conversion. The highest butanol concentration obtained was 17 g/l (not listed in Table 2).

Fermentation with molecular nitrogen and hydrogen: *C. pasteurianum* is an anaerobe that readily fixes nitrogen. This ability was verified also for glycerol in a medium containing only 0.1 g yeast extract/l. As expected, the fermentation time was longer than that of an equally inoculated ammonium-grown culture. The product spectrum was shifted to 1,3-propanediol and butyrate, and a decrease in lactate formation was found (Table 3). Two repetitions gave similar results.

In one fermentation, hydrogen was used as sparging gas in order to check its influence on the fermentation balance. No significant difference to the nitrogen-sparged parallel culture was found (not shown).

Growth in a chemically defined medium: Strain DSM 525 was able to grow and ferment glycerol in a mineral medium supplemented only by biotin, but it lasted three times as long as an equally inoculated culture with 1 g yeast extract/l. The final product composition was almost the same in the defined medium and that containing yeast extract (Table 4).

Continuous culture

Chemostat at varied dilution rates: A continuous fermentation fed with a medium containing 36 g glycerol/l is shown in Figure 3 for dilution rates between 0.1 and 0.4 h^{-1}. The culture was limited by glycerol only at the lowest dilution rate; at faster medium flow, an increasingly smaller fraction was fermented, probably due to inhibition by the products. 1,3-Propanediol was the main product at 0.1 h^{-1}, while butanol and ethanol were highest at the higher dilution rates. In contrast to the batch fermentations (Tables 2–4), appreciable amounts of ethanol were also formed at pH 6.0 parallel to butanol.

Chemostat at varied glycerol concentrations: When the chemostat was run at a constant dilution rate of 0.32 h^{-1} and glycerol concentration was increased stepwise (Figure 4), linearly increased glycerol amounts were consumed up to a concentration of 350 mmol/l (30 g/l). At higher concentrations, glycerol consumption remained on the same level and decreased above 800 mmol/l. At no steady state was the substrate used up completely. Even in the linear phase, only about 40% of the substrate was consumed. Most of the glycerol was fermented to butanol and ethanol, but propanediol formation increased with increasing glycerol concentration.

Fermentation in a pH auxostat: In contrast to a chemostat, a pH auxostat is run at an excess of nutrients. The medium is supplied in the periods in which the pH is corrected by alkali addition. In the version used, alkali flow was varied at a constant medium flow, i.e.,

Table 1 Influence of pH on fermentation time and product formation

pH	Fermentation time [h]	Product yields [mol/100 mol glycerol]					Recovery [%]
		Butanol	1,3-Pd	Ethanol	Butyrate	Acetate	
4.5	<50	38.8	9.1	2.4	0.2	2.3	92
5.0	22	23.1	10.8	1.3	7.2	2.2	75
5.5	24	17.7	22.5	1.3	10.6	3.9	84
6.0	21	32.4	5.1	4.5	2.9	1.3	82
6.5	20	23.5	19.1	7.2	1.1	2.4	78
7.0	21	20.0	20.9	13.9	0.1	4.1	79
7.5	20	22.2	5.8	26.5	0.2	0.2	77

Glycerol concentration, 50 g/l.

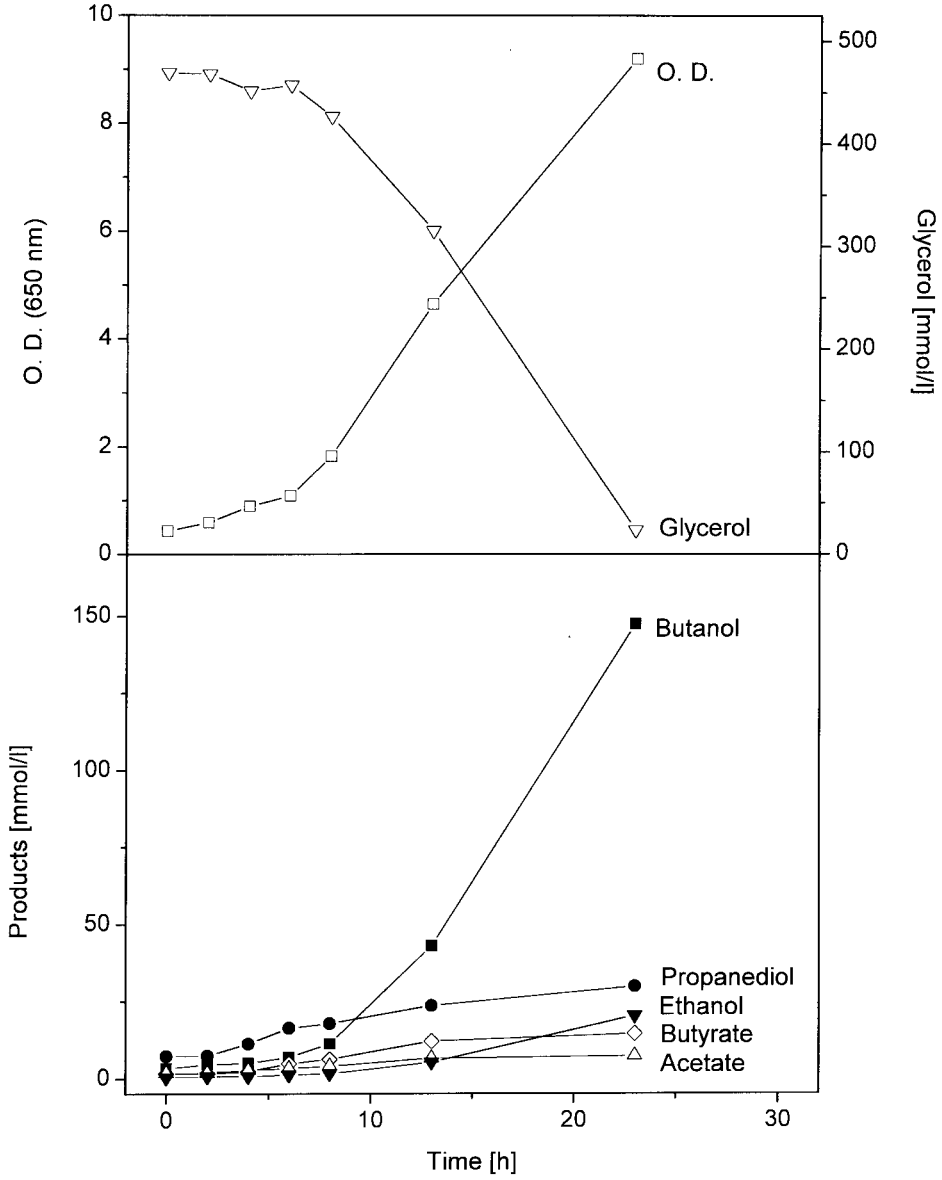

Figure 2 Growth, glycerol consumption and product formation in a batch fermentation with 5% glycerol at pH 6.0.

low alkali flow rates lead to long periods of medium input and therefore to a high medium flow and *vice versa*. The glycerol concentration was increased according to the alkali flow rate to avoid substrate limitation, which would stop alkali addition and medium supply. As expected, cell density and glycerol consumption increased in proportion to the alkali supply while the resulting dilution rate decreased with product accumulation (Figure 5). The product formation in this kind of continuous culture differs from

Table 2 Glycerol utilization and product yield at increasing glycerol concentration and under fed-batch conditions (Fb)

Initial glycerol [g/l]	Glycerol utilized [g/l]	Product yield [mol/100 mol glycerol]						Recovery [%]
		Butanol	1,3-Pd	Ethanol	Butyrate	Acetate	Lactate	
29.5	29.5	17.1	26.4	1.5	13.1	3.2	1.3	95
54.2	53.1	27.0	10.5	2.5	3.9	1.2	n.d.	76
83.7	62.4	18.0	23.4	3.6	3.9	3.6	3.2	79
114.6	63.6	28.1	10.5	4.2	3.3	4.2	1.2	83
Fb	57.1	31.8	11.3	4.9	1.7	1.1	n.d.	84

The fed-batch culture was set up with an initial glycerol concentration of 50 g/l; 6.0 g/l was added after 14 h, 10.5 g/l after 25 h.

Table 3 Influence of the nitrogen source on fermentation time and product yield

Nitrogen source	Fermentation time [h]	Product yield [mol/100 mol glycerol]						Recovery [%]
		Butanol	1,3-Pd	Ethanol	Butyrate	Acetate	Lactate	
NH_4^+	37	18.4	26.4	2.9	2.3	0.9	18.3	90
N_2	49	15.0	37.4	0.8	10.3	1.2	8.1	107

Medium with 50 g glycerol and 0.1 g yeast extract per liter, sparged with nitrogen; N_2 as nitrogen source means: a bound nitrogen source was not added.

that of the chemostat shown in Figure 3 in that 1,3-propanediol and lactate are the main products in all steady states, with butanol and ethanol being byproducts. Only 34% of the glycerol consumed was fermented to butanol and ethanol compared to 85% in the chemostat culture (D=0.22 h^{-1}).

Discussion

When fermenting glycerol, *C. pasteurianum* is able to form a relatively great variety of products including butanol, ethanol, 1,3-propanediol, butyric, acetic and lactic acids. Under the majority of conditions tested in this investigation, i.e., batch and chemostat cultures, butanol was the main product sometimes accompanied by ethanol. In pH auxostat cultures, in which all nutrients are in excess, 1,3-propanediol was the dominant product in addition to lactic acid. In some batch cultures (Tables 1–4), the molar yield of 1,3-propanediol also exceeded that of butanol, but due to the fermentation stoichiometry in all these fermentations, more glycerol was converted to butanol than to 1,3-propanediol.

The following considerations are to show that, indeed, fermentation to butanol is the energetically preferred pathway, but that formation of 1,3-propanediol is necessary for the reducing equivalent balance in any case. The reaction equation of glycerol fermentation to butanol or ethanol shows a redox neutral conversion, which does not require formation of byproducts:

$$2\ CH_2OH-CHOH-CH_2OH \rightarrow CH_3-CH_2-CH_2-CH_2OH + 2\ CO_2 + 2\ H_2 + H_2O \quad (1a)$$

$$CH_2OH-CHOH-CH_2OH \rightarrow CH_3-CH_2OH + CO_2 + H_2 \quad (1b)$$

However, as glycerol is more reduced than the cell mass formed along with the fermentation products, additional reducing equivalents are released and need an acceptor. That is why a certain amount of glycerol always has to be reduced to 1,3-propanediol. If a cell mass formula of $C_4H_7O_2N$ is used, cell mass formation from glycerol can be formulated as:

$$\underset{\text{glycerol}}{4\ C_3H_8O_3} + NH_3 \rightarrow \underset{\text{cell mass}}{3\ C_4H_7O_2N} + 8\ [H] + 6\ H_2O \quad (2a)$$

$$\underset{\text{glycerol}}{4C_3H_8O_3} + 8\ [H] \rightarrow \underset{1,3-\text{propanediol}}{4\ C_3H_8O_2 + 4\ H_2O} \quad (2b)$$

$$8\ C_3H_8O_3 + NH_3 \rightarrow 3\ C_4H_7O_2N + 4\ C_3H_8O_2 + 10\ H_2O \quad (2)$$

If an ATP requirement of 36 mol is used for Equation 2a and an ATP production of 2 mol in Equation 1 (see Refs. [12,13]), the complete reaction equation for glycerol fermentation to butanol can be written as:

$$44\ \text{glycerol} + 3\ NH_3 \rightarrow 3\ C_4H_7O_2N + 18\ \text{butanol} + 4\ 1,3-\text{propanediol} + 36\ CO_2 + 36\ H_2 + 10\ H_2O$$

showing that at least 4/44=9% (mol/mol) of the glycerol has to be converted to 1,3-propanediol. For the other fermentation pathways, i.e., to ethanol, and to butyric, acetic and lactic acids, corresponding equations can be formulated. The resulting yields for these products, for 1,3-propanediol and for biomass have been expressed as glycerol required for their formation and are listed in Table 5. They show that highest biomass is obtained in glycerol fermentations to butanol and ethanol, which might well explain the preference for these products. The lowest biomass is formed if 1,3-propanediol and lactate are produced from glycerol, which has been found in the pH auxostat steady states.

Zeng *et al* [14] established a model for growth and product formation in 1,3-propanediol-producing organisms. They suggested that the preference for a particular pathway depends on the source of limitation. If the carbon source is the limiting factor, the pathway is used, which yields a maximum of energy, e.g., butyric acid in *C. butyricum* and ethanol in *Klebsiella pneumoniae* instead of 1,3-propanediol and acetic acid. When the carbon source is in excess, by limitation through inorganic nutrients or product inhibition, products are formed that cause the least inhibition, i.e., 1,3-propanediol and acetic acid instead of butyric acid or ethanol. The pH auxostat culture is grown at

Table 4 Growth and product formation in a chemically defined medium with 30 g glycerol/l containing biotin compared to a medium with yeast extract

Growth factor	Fermentation time [h]	Product yield [mol/100 mol glycerol]						Recovery [%]
		Butanol	1,3-Pd	Ethanol	Butyrate	Acetate	Lactate	
Biotin, 25 μg/l	59	17.4	29.2	1.6	12.1	3.3	7.5	101
Yeast extract, 1 g/l	19	17.1	26.5	1.5	12.6	3.2	1.3	95

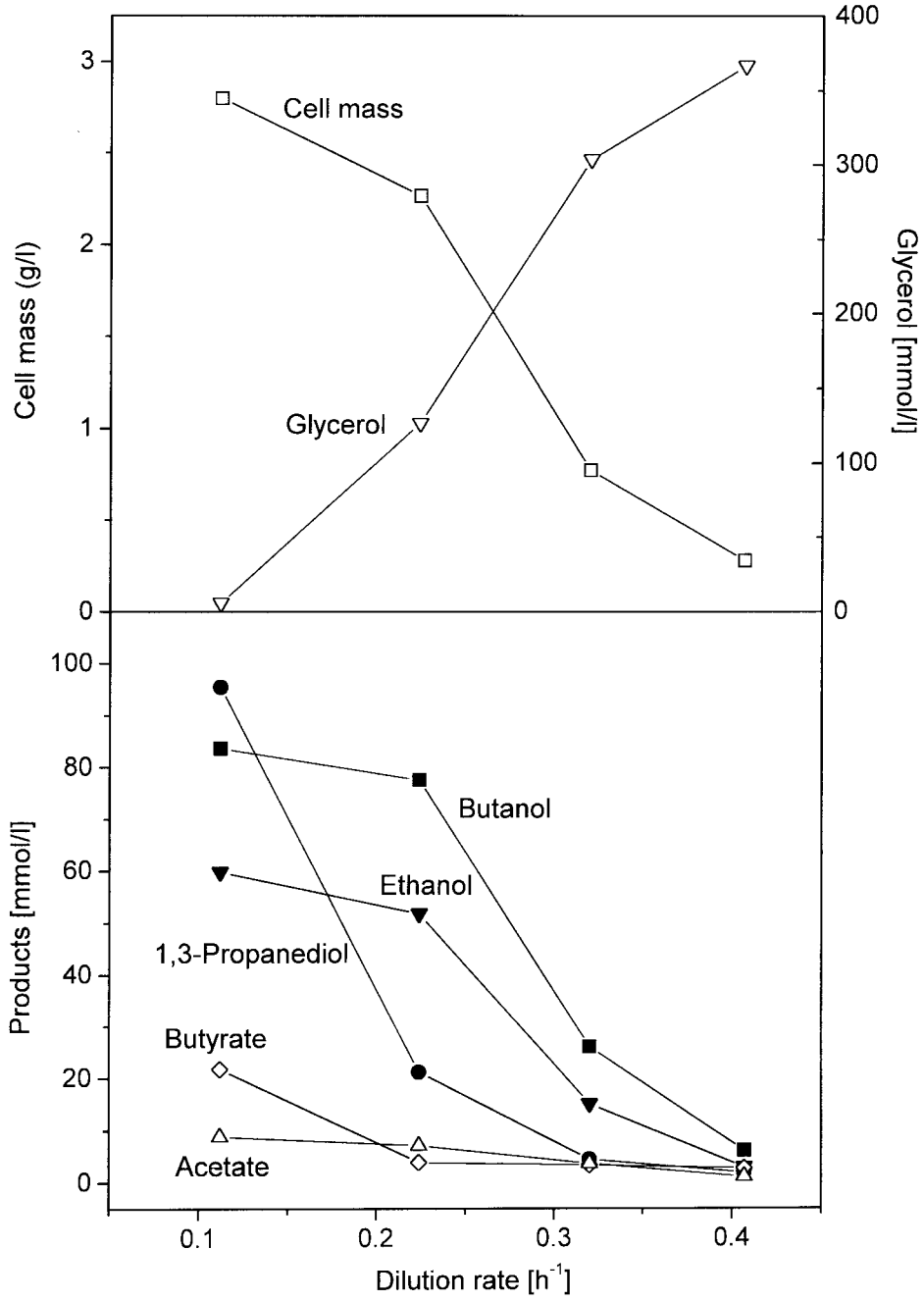

Figure 3 Cell mass, glycerol consumption and products in a chemostat culture at increasing dilution rate. Glycerol feed concentration, 36 g/l.

a permanent glycerol excess, but also under conditions of product inhibition as is obvious from the decreasing growth rate, and indeed 1,3-propanediol, the least toxic of all products, is formed as the main product. The chemostat cultures of *C. pasteurianum* that generally form more butanol than 1,3-propanediol do not fit well into this model as in most of the steady states substrate excesses were observed. It appears that other factors than energy yield and product inhibition interfere in this fermentation.

The alternative or simultaneous glycerol fermentation to butyric and acetic acids (and much 1,3-propanediol) or butanol and ethanol (and little 1,3-propanediol) is reminiscent of the fermentation of glucose by *C. acetobutylicum*, which forms butanol and acetone in addition to or instead of butyric and acetic acid. As in *C. acetobutylicum* batch cultures, butanol is not produced from the beginning, but only after some accumulation of butyric and acetic acids (and 1,3-propanediol). The factors that have been found to trigger butanol and acetone formation in *C. acetobutylicum* are numerous and comprise pH, butyrate concentration, initial substrate concentration, growth rate and physiological state of the cells used for inoculation [2], and at times it was difficult to predict reliably

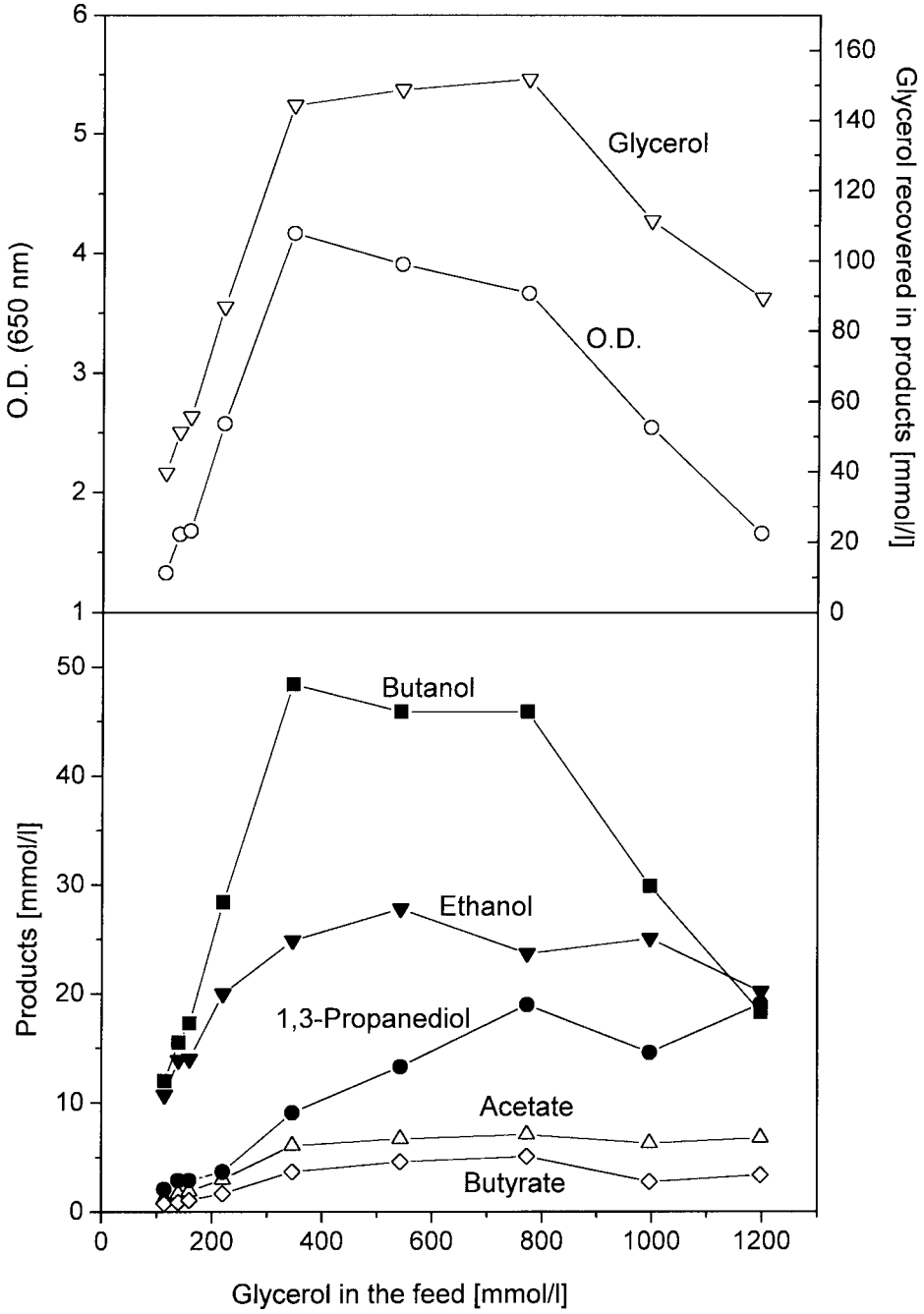

Figure 4 Cell mass, glycerol consumption and product formation in a chemostat culture with increasing glycerol feed concentration and constant dilution rate ($0.32\ h^{-1}$).

whether the transition from acid to solvent production would occur or not, particularly in the industrial process [7]. In the glycerol fermentation of *C. pasteurianum*, there might be a regulation which is similarly complex and subject to several interdependent environmental influences. In contrast to *C. acetobutylicum*, product formation of *C. pasteurianum* is not significantly affected by the pH, confirming the observation of Dabrock *et al* [5] in continuous culture with glucose as substrate.

Particularly in batch cultures, a high variability in product formation was observed. When examining the influence of pH between 5 and 7.5, of glycerol concentration and of the presence of yeast extract, there were considerable fluctuations in the product pattern within the test series (Tables 1 and 2). It appears that small variations in the culture conditions or slight differences in the precultures were able to change the product selectivity considerably. Such a weak regulation between the fermentation pathways seems to be a general feature of this fermentation. It has also been observed in a number of other continuous cultures not presented here. In one of them, the culture switched from an almost pure butanol fermentation to an almost pure 1,3-propanediol/acids fermentation when the

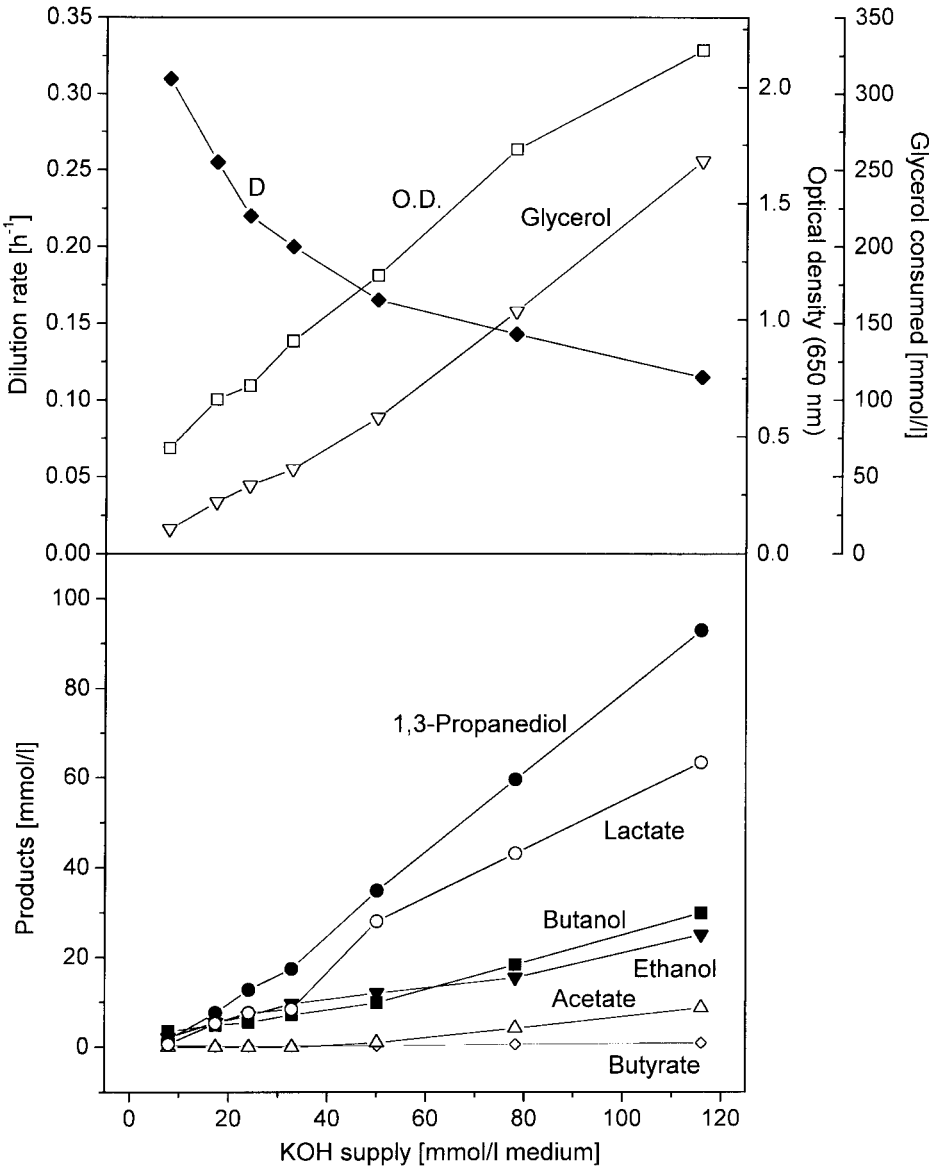

Figure 5 Cell mass, glycerol consumption and product formation in a pH auxostat culture at a varied alkali/medium supply ratio. Glycerol feed adjusted to a concentration of about 20% above the expected consumption.

dilution rate was changed from 0.1 to 0.2 h^{-1}. Xiu *et al* [11] recently calculated for the glycerol fermentation of *K. pneumoniae* that, under defined conditions, multiple steady states at one and the same dilution rate are possible. Thus, apparently insignificant changes in the culture conditions could give rise to a leap into another steady state.

Table 5 Fermentation balances in case only one product other than 1,3-propanediol is formed

Product of the energy-generating pathway	Product yield [mol% of glycerol used for product formation]		
	Product	1,3-Pd	Biomass*
Butanol	81.8	9.1	9.1
Ethanol	81.8	9.1	9.1
Butyrate	42.7	50.0	7.1
Acetate	28.9	64.5	6.6
Lactate	45.0	50.0	5.0

Calculated according to Ref. [12].
*Assuming that 4/3 mol glycerol is required for 1 mol biomass of the formula $C_4H_7O_2N$.

C. pasteurianum is the only known organism that is able to ferment glycerol to butanol. Due to formation of biomass, which is less reduced than glycerol, a small amount of 1,3-propanediol also has to be produced. *C. pasteurianum* DSM 525 can also ferment glycerol to 1,3-propanediol and acids as does *C. butyricum* [3], but more often a mixture with varying fractions of all these products is found. Under most conditions, more glycerol is fermented to butanol than to 1,3-propanediol probably because of the higher energy yield, which is obtained in butanol formation. The preference for 1,3-propanediol in the pH auxostat cultures is explained by substrate excess and product inhibition conditions that result in formation of the least toxic product. The great variation in product formation in batch culture under equal or slightly different conditions is seen as an expression of weak pathway regulation and multiplicity effects.

Both of the alternative main products of the glycerol fermentation of *C. pasteurianum* are of industrial interest as bulk chemicals, butanol as a general solvent, and 1,3-propanediol as a monomer for polyesters. Butanol has been produced from starch or molasses by *C. acetobutylicum* in the classical butanol–acetone process, which resulted in about 12 g butanol, 6 g acetone and 2 g ethanol per liter. In recent research, the butanol concentration was increased to 20 g/l using conventional and genetically engineered mutants. The maximum butanol concentration obtained with *C. pasteurianum* from glycerol was 17 g/l, indicating that product tolerance is about the same in both processes. However, due to a faster growth rate in batch culture (1 day *versus* 2 days for 6% of substrate), *C. pasteurianum* has a higher substrate conversion rate, and due to lower byproduct formation (no acetone formed), also a better product yield. The disadvantage of the *C. pasteurianum* fermentation is the relatively high cost of glycerol, unless cheaper carbohydrates can be made available *via* genetic engineering. Perhaps it can be said that, if the butanol–acetone fermentation can be revived as an industrial process, the butanol fermentation of *C. pasteurianum* should also be considered. 1,3-Propanediol is presently chemically synthesized, but the biotechnological process is seriously discussed [4] particularly for recombinant strains able to utilize carbohydrates. Here, *C. pasteurianum* appears less suitable than other glycerol-fermenting microorganisms regarding the great variety of possible byproducts.

From the physiological point of view, the fermentation described is interesting as it combines the two pathways of butanol and of 1,3-propanediol formation in one organism. The latter pathway serves to regenerate NADH, which is released during biomass formation but may also become the major route in the anaerobic utilization of glycerol by this species.

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