Kinetic, Dynamic, and Pathway Studies of Glycerol Metabolism by *Klebsiella pneumoniae* in Anaerobic Continuous Culture: III. Enzymes and Fluxes of Glycerol Dissimilation and 1,3-Propanediol Formation

K. Ahrens, K. Menzel, A.-P. Zeng, W.-D. Deckwer

GBF-Gesellschaft für Biotechnologische Forschung mbH, Biochemical Engineering Division, Mascheroder Weg 1, D-38124 Braunschweig, Germany; telephone: +49-531-6181-188; fax: +49-531-6181-111; e-mail: AZE@GBF.de

Received 18 July 1997; accepted 22 January 1998

Abstract: The initial steps of glycerol dissimilation and 1,3-propanediol (1,3-PD) formation by *Klebsiella pneumoniae* anaerobically grown on glycerol were studied by quantifying the in vitro and in vivo activities of enzymes in continuous culture under conditions of steady state and oscillation and during transient phases. The enzymes studied included glycerol dehydrogenase (GDH), glycerol dehydratase (GDHt), and 1,3-propanediol oxidoreductase (PDOR). Three conclusions can be drawn from the steady-state results. First, glycerol concentration in the culture is a key parameter that inversely affects the in vitro activities (concentrations) of all three enzymes, but has a positive effect on their in vivo activities. Growth rate significantly affects the ratio of in vitro and in vivo enzyme activities under low glycerol concentrations, but not under glycerol excess. Second, whereas the flux through the oxidative pathway of glycerol dissimilation is governed mainly by the regulation of in vivo enzyme activity on a metabolic level, the flux through the reductive pathway is largely controlled by the synthesis of enzymes. Third, GDHt is a major rate-liming enzyme for the consumption of glycerol and the formation of 1,3-PD in *K. pneumoniae* at high glycerol concentrations. Results from oscillating cultures revealed that both in vitro and in vivo activities of the enzymes oscillated. The average values of the in vitro activities during an oscillation cycle agreed well with their corresponding values for nonoscillating cultures under similar environmental conditions. Experiments with step changes in the feed concentration of glycerol demonstrated that growth and product formation are very sensitive to changes of substrate concentration in the culture. This sensitivity is due to the dynamic responses of the genetic and metabolic networks. They should be considered when modeling the dynamics of the culture and attempting to improve the formation of 1,3-PD. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **59:** 544–552, 1998.

Keywords: *Klebsiella pneumoniae;* glycerol dissimilation; 1,3-propanediol; in vitro and in vivo enzyme activities; dynamics

Correspondence to: A.-P. Zeng

INTRODUCTION

Under anaerobic conditions glycerol is dissimilated in *Klebsiella pneumoniae* through coupled oxidative and reductive pathways (Fig. 1) (Lin, 1976). The oxidation of glycerol is catalyzed by glycerol dehydrogenase (GDH), which leads to the formation of dihydroxyacetone (DHA) under the generation of reducing equivalent ($NADH_2$). Glycerol kinase is another enzyme for the oxidation of glycerol, which is only active under aerobic conditions (Forage and Lin, 1982). Dihydroxyacetone formed by GDH is further metabolized to various products such as ethanol and acetic acid through pyruvate. The reducing power is normally regenerated in the reductive pathway of glycerol dissimilation. The latter consists of two enzyme reactions. First, glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (GDHt). 3-HPA is then converted to 1,3-propanediol (1,3-PD) by the enzyme 1,3-propanediol oxidoreductase (PDOR) under the oxidation of $NADH_2$. Because of the appealing properties of 1,3-PD as a monomer for the production of polyesters and heterocycles the fermentative production of 1,3-PD has received much attention in the past 10 years (Zeng et al., 1997) and has recently been industrially commercialized (Stinson, 1995; see also press releases of the companies DuPont, 1996, and Shell, 1995). From an economical point of view the yield of 1,3-PD from the substrate and the final PD concentration are two important parameters for process optimization (Deckwer, 1995). To this end, a quantitative understanding of key factors that influence the enzyme activities and metabolic fluxes is desired, as well as an identification of limiting step(s) of the pathways. This kind of information will be also useful for explaining the different performances of microorganisms so far extensively studied for the microbial production of 1,3-PD from glycerol (Homann et al., 1990; Zeng, 1996). It has been reported that the reaction catalyzed by GDHt is the limiting step for 1,3-PD formation in *Citrobacter freundii*

CCC 0006-3592/98/050544-09

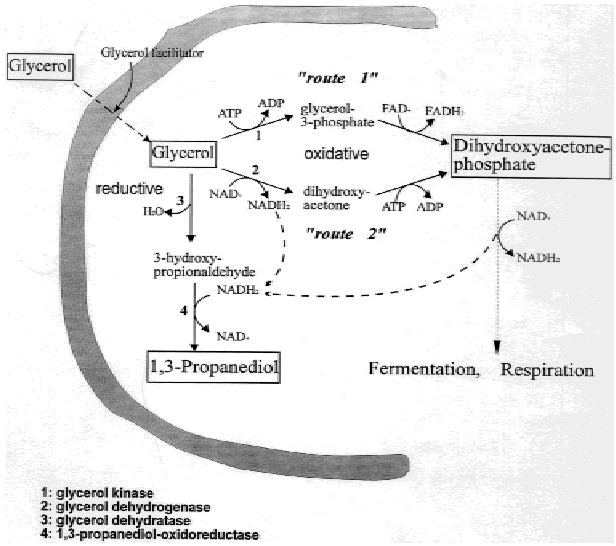

Figure 1. Initial steps of glycerol dissimilation and 1,3-propanediol formation in *Klebsiella pneumoniae.*

(Boenigk et al., 1993) and *Clostridium butyricum* (Abbad-Andaloussi et al., 1996). No information in this regard is available for *K. pneumoniae* that achieves a higher productivity in continuous culture than both *C. freundii* and *C. butyricum* (Menzel et al., 1997a).

Another noteworthy aspect of the glycerol metabolism of *K. pneumoniae* is the dynamic behavior of this microorganism in continuous culture (Menzel et al., 1996; Zeng et al., 1996). Sustained oscillations and multiplicity of growth and product formation have been observed under a variety of conditions. These phenomena revealed several different patterns and new features compared with those reported in the literature. They were shown to be bound to genuine fluctuations of the metabolic pathways of the microorganism triggered by substrate excess and drastic change(s) of environmental conditions (Menzel et al., 1996). Further analysis of the metabolic fluxes under steady-state and oscillation conditions indicated that the instability is mainly encountered in the pyruvate metabolism, whereas the fluxes of the initial steps of glycerol dissimilation and 1,3-PD formation appeared to be less significantly affected (Zeng et al., 1996). However, it is not known how the metabolic fluxes are regulated on enzyme levels, particularly under conditions of oscillation and hysteresis.

In this work, we report on experimental results concerning the effects of culture conditions on the in vitro and in vivo activities of glycerol dehydrogenase, glycerol dehydratase, and 1,3-propanediol oxidoreductase under both steady-state and oscillation conditions. The genes for these three enzymes and the enzyme dihydroxyacetone kinase are encoded in one and the same regulon named *dha* (Forage and Lin, 1982; Tong et al., 1991) and have been cloned and sequenced for *K. pneumoniae* (Cameron et al., 1998; Tong et al., 1991) and *C. freundii* (Daniel and Gottschalk, 1992; Daniel et al., 1995). The transient behavior of the culture after step changes of substrate concentration in medium is also studied. The in vitro and in vivo activities of enzymes are compared to gain quantitative information on the regulation of the metabolic fluxes and to identify possible limiting step(s). In an accompanying article, experimental results concerning the enzymes associated with the formation and metabolism of pyruvate and acetyl-CoA in *K. pneumoniae* are presented (Menzel et al., 1997c).

MATERIALS AND METHODS

Strain and Culture Medium

Klebsiella pneumoniae DSM 2026 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) was used in this study. Culture medium composition was previously reported by Menzel et al. (1997b). Glycerol solution (87% by weight) was separately autoclaved and added to the medium reservoir according to the glycerol concentration required in the feed medium.

Fermentation Setup and Analytical Methods

All fermentations were carried out in a 4-L Setric bioreactor (Set 4V, Setric Genie Industriel, Toulouse, France) with a working volume of 2 L. The fermentation setup and the analytical methods have been described by Menzel et al. (1996). The fermenter was additionally equipped with a fast sampling device (Pec Tec, Mühlacker, Germany) and was connected to a real-time computer control system (Ubicon, ESD, Hannover, Germany) for on-line data acquisition. The cultivation conditions for all fermentations were as follows: temperature 37°C, pH 7.0 (by automatic control using 20% NaOH), and 300 rpm for agitation. To ensure anaerobic conditions the bioreactor was sparged with nitrogen at a flow rate of 0.4 volume per volume per minute. Beginning with a relatively low glycerol concentration in the medium, which resulted in substrate limitation in the reactor, the substrate concentration was carefully increased by small steps until glycerol was in excess in the culture. At each dilution rate, steady states were obtained in this way at different glycerol concentrations in the medium.

Preparation of Cell Free Extract

Culture samples for enzyme assays were taken anaerobically with a fast sampling device in −20°C precooled tubes flushed with nitrogen. The samples were rapidly cooled on ice. All preparation steps afterwards and the determination of enzyme activities were done in an anaerobic chamber as described by Menzel et al. (1997b). After cell disruption all steps were carried out under strict exclusion of light because GDHt can form an irreversible complex with the coenzyme B_{12} in the presence of light and is inactivated in this way. After pretreatment the eluate was immediately assayed for enzyme activities. Protein concentration was determined according to Lowry et al. (1951).

Enzyme Assays

Determination of glycerol dehydrogenase (EC 1.1.1.6, glycerol:NAD^+ 2-oxidoreductase) activity was done as described by Ruch et al. (1974). The reaction mixture (1 mL) contained 30 m*M* ammonium sulfate, 0.2 *M* glycerol, 1.2 m*M* NAD (adjusted to pH 7.0 with 1 *M* NaOH), and the eluate with enzymes in 0.1 *M* potassium carbonate buffer solution (pH 9.8). The assay was initiated by adding the cell extract, and the absorption increase ($NADH_2$ formation) was followed with a spectrophotometer (Novospec II, Pharmacia Biotech, Freiburg, Germany) at 340 nm and 30°C. One unit of activity is the amount of enzyme required to reduce 1 μmol of substrate per minute under the conditions specified. Specific enzyme activity (as units per milligram of protein) was extrapolated to the fermentation temperature of 37°C using a correction factor of 1.92, according to a calibration curve with pure enzyme (Ahrens, 1997).

Two methods are generally used to determine the activity of glycerol dehydratase (EC 4.2.1.30, glycerol hydrolyase). The method of Yakusheva et al. (1974) couples the glycerol dehydration reaction to a reduction of the aldehyde formed with alcohol dehydrogenase. A more sensitive method is based on the reaction of aldehyde with 3-methyl-2-benzothiazolinone-hydrazone (MBTH) (Abbad-Andaloussi et al., 1996; Forage and Foster, 1979; Honda et al., 1980; Toraya and Fukui, 1977; Toraya et al., 1976). The latter is a so-called endpoint measurement after a dehydration time of about 10 min, and is widely used. A comparison of these two methods with cell extract of *K. pneumoniae* showed that the MBTH method generally gave 15% to 20% higher activity than the method of Yakusheva (Ahrens, 1997). This was mainly because of the inhibition of glycerol on GDHt, which caused complete cessation of the GDHt activity within about 3 min (Toraya et al., 1976). The inactivation by glycerol also hinders direct application of the MBTH method. To overcome this problem, another substrate of GDHt, 1,2-propanediol, is normally used for the enzyme assay. It has been shown that the initial velocity of dehydration with glycerol is comparable to that with 1,2-PD (Honda et al., 1980; Toraya et al., 1976). For this reason, we also used 1,2-PD as substrate for the assay of GDHt. The reaction was carried out under strict exclusion of light. The assay mixture contained (in a total volume of 1 mL) 0.05 *M* KCl, 0.2 *M* 1,2-propanediol, 15 μ*M* coenzyme B_{12}, and eluate in 0.035 *M* potassium phosphate buffer solution (pH 7.0). The assay was started by the addition of cell extract and incubated at 37°C. After 10 min incubation, the reaction was stopped by adding 1 mL of 0.1 *M* potassium citrate buffer (pH 3.6). For developing the color after adding 0.5 mL of 0.1% MBTH solution the mixture was incubated again for 15 min at 37°C. The colored azin ($\varepsilon = 13.22$ L $mmol^{-1}$ cm^{-1}) formed was detected after addition of 1 mL of distilled water at 305 nm. For all samples repeated determinations were done with different eluate concentrations. To convert the 1,2-PD-dehydrating activity to glycerol-dehydrating activity we estimated the ratio of glycerol-dehydrating and 1,2-PD-dehydrating activities by triplicate 1 min assays with both glycerol and 1,2-PD as substrates, according to Yakushewa et al. (1974) and Toraya and Fukui (1977). For cell extract of *K. pneumoniae* this ratio was determined to be 1.41 at pH 7.0.

The activity of 1,3-PD oxidoreductase (EC 1.1.1.202) is usually assayed using the reverse reaction rather than the physiological one due to the instability of 3-HPA, which is also commercially unavailable (Abbad-Andalousi et al., 1995; Forage and Foster, 1982; Johnson and Lin, 1987). According to Forage and Foster (1982), the reaction mixture contained, in a volume of 1 mL 30 m*M* ammonium sulfate, 0.1 *M* 1,3-propanediol, 2 m*M* NAD (adjusted to pH 7.0 with 1 *M* NaOH), and eluate in 0.1 *M* potassium carbonate buffer solution (pH 9.0). The reaction was started by adding cell extract and the increase of $NADH_2$ was followed photometrically at 340 nm with a spectrophotometer. Specific enzyme activity was extrapolated to the fermentation temperature of 37°C using a correction factor of 1.82 according to a calibration curve with eluated enzyme (Ahrens, 1997). To estimate the ratio of in vitro PDOR activities for the reverse and physiological reactions we used propionaldehyde, which is known to be a proper substrate of PDOR (Daniel et al., 1995). Daniel et al. (1995) showed with PDOR from *C. freundii* that propionaldehyde reached 21% of the activity of 3-HPA. With PDOR from *K. pneumoniae* we determined the ratio of reduction velocity of propionaldehyde to dehydration velocity of 1,3-PD to be 0.83 at pH 7.0. Combining the results of Daniel et al. (1995) we used a conversion factor of $0.83/0.21 = 3.95$ to convert the specific activity of PDOR from the reverse reaction to the physiological reaction.

Calculations

In Vitro Activities of Enzymes

The in vitro activities of enzymes on the basis of biomass were calculated as follows:

$$\text{Enzyme}_{\text{in vitro}} = \text{Enzyme}_{\text{spec}} \times Y_{\text{protein/biomass}}$$

where $\text{Enzyme}_{\text{spec}}$ is the specific enzyme activity based on protein (moles per hour per gram of protein) by the assay methods just described, and $Y_{\text{protein/biomass}}$ the ratio of protein content of the cell extraction to total dry biomass (grams per gram).

In Vivo Activities of Enzymes

The in vivo activities of enzymes were calculated based on the metabolic fluxes measured and the stoichiometry of the pathways as follows:

$$\text{GDH}_{\text{in vivo}} = q_{\text{glyc}} - q_{\text{PD}}$$

$$\text{GDHt}_{\text{in vivo}} = q_{\text{PD}}$$

$$\text{PDOR}_{\text{in vivo}} = q_{\text{PD}}$$

where q_{glyc} is the specific glycerol uptake rate (millimoles per gram per hour), and q_{PD} the specific formation rate of 1,3-propanediol (millimoles per gram per hour).

RESULTS AND DISCUSSION

In Vitro and In Vivo Enzyme Activities Under Steady States

Continuous cultures were carried out at five different dilution rates between 0.13 and 0.5 h^{-1} under varied glycerol concentrations ranging from limitation to large excess in the culture. The concentrations of biomass, substrate, and products were measured at steady states as well as the enzyme activities. The concentrations, metabolic rates, and yields were similar to those reported by Zeng et al. (1996) and Menzel et al. (1996). In what follows, only the results of enzyme assays are presented and discussed.

Figure 2A shows the specific GDH activities (GDH_{spec}) at different dilution rates as a function of glycerol concentration (C^{Rct}_{Glyc}) in the culture. Surprisingly, GDH_{spec} declined with increasing C^{Rct}_{Glyc} at all dilution rates, particularly at low glycerol concentrations. Under conditions of obvious glycerol excess GDH_{spec} approached relatively constant values between 2.5 and 5 U/mg protein. GDH_{spec} was also a function of the dilution rate (D), decreasing with increasing dilution rate. Such a behavior of the enzyme is not expected in view of the fact that the glycerol flux supported by this enzyme increased with C^{Rct}_{Glyc} (Zeng et al., 1996). The decrease of GDH_{spec} with D at identical C^{Rct}_{Glyc} values is also unexpected because a higher flux of the glycolysis is needed for biosynthesis at a higher growth rate. The need of glycolysis flux for biosynthesis may be better reflected by the relationship between GDH_{spec} and the specific uptake rate of glycerol (q_{Glyc}) at different dilution rates (Fig. 2B). Because the uptake rate of glycerol (q_{Glyc}) is a strong function of the residual glycerol concentration in this strain (Zeng and Deckwer, 1995) it can be inferred from Figure 2A that the in vitro activity of GDH also decreased with increasing q_{Glyc}, as depicted in Figure 2B. In contrast to Figure 2A, however, GDH_{spec} proved to increase with increasing D at identical q_{Glyc} values. For all dilution rates, GDH_{spec} was very sensitive to q_{Glyc} at low q_{Glyc} values (glycerol limitation), but reached a relatively constant value (about 2.5 U/mg protein) at high q_{Glyc} values. At a similar q_{Glyc} value in the low range GDH_{spec} rapidly increased with D, whereas, at high values of q_{Glyc} (substrate excess), the dependence of GDH_{spec} on D diminished. This is probably due to the fact that q_{Glyc} is a strong function of growth rate under conditions of substrate limitation, yet it is determined mainly by glycerol concentration under conditions of substrate excess (Zeng and Deckwer, 1995). In general, it can be stated from Figure 2A and B that the specific in vitro GDH activity (i.e., the synthesis of GDH) is not closely coupled with the glycerol uptake rate. The specific activity of GDH measured in this work is quite high compared with those reported for *K. pneumoniae* (Ruch et al., 1974) and other microorganisms such as *Aerobacter aerogenes* (McGregor et al., 1974), *Hansenula ofunaensis* (Yamada and Tani, 1988), and *C. butyricum* (Abbad-Andaloussi et al., 1996). The reported values were in the range of 0.4 to 2.4 U/mg protein, which are close to those measured for conditions of strong substrate excess in this work (Fig. 2). It seems that the effect of substrate concentration or uptake rate as shown in this work has often been overlooked in the literature.

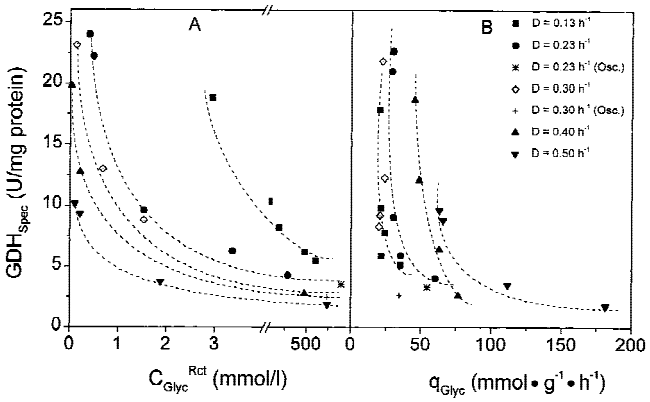

Figure 2. Specific activity of glycerol dehydrogenase (GDH) as a function of glycerol concentration in the reactor (C^{Rct}_{Glyc}) (A) and specific glycerol uptake rate (q_{Glyc}) (B) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

Another important issue is that the in vitro enzyme activity measured under optimum assay conditions gives only information on the maximum possible enzyme activity or the enzyme concentration. A comparison of the in vitro and in vivo activities can give useful information on the intracellular regulation of synthesis and activity of the enzyme. Figure 3A shows the ratio of in vitro and in vivo activities of GDH ($GDH_{in\ vitro/in\ vivo}$) as a function of C^{Rct}_{Glyc} for different dilution rates. At low glycerol concentrations, $GDH_{in\ vitro/in\ vivo}$ reached values as high as 30 to 50 at high growth rates and 100 at a dilution rate of 0.23 h^{-1}, indicating that only a small portion of the catalysis capacity of the enzyme was utilized, due to either substrate limitation or inhibition of the enzyme by inhibitors such as $NADH_2$ and DHA (McGregor et al., 1974). $GDH_{in\ vitro/in\ vivo}$ significantly decreased with increasing C^{Rct}_{Glyc} and reached values as low as 2 at high residual glycerol concentrations and high growth rate, implying an increased utilization of the enzyme synthesized under these conditions. In general, it may be stated that the flux from glycerol to DHA is not limited by the synthesis rate of GDH, but rather by substrates and/or different effectors of this enzyme.

The effects of glycerol concentration and dilution rate on the in vitro activities of GDHt and PDOR are summarized in Figures 4 and 5, respectively. Although the specific activities of these two enzymes were much lower compared with the specific activity of GDH they showed similar trends with respect to the effects of C^{Rct}_{Glyc} and D. Quantitatively,

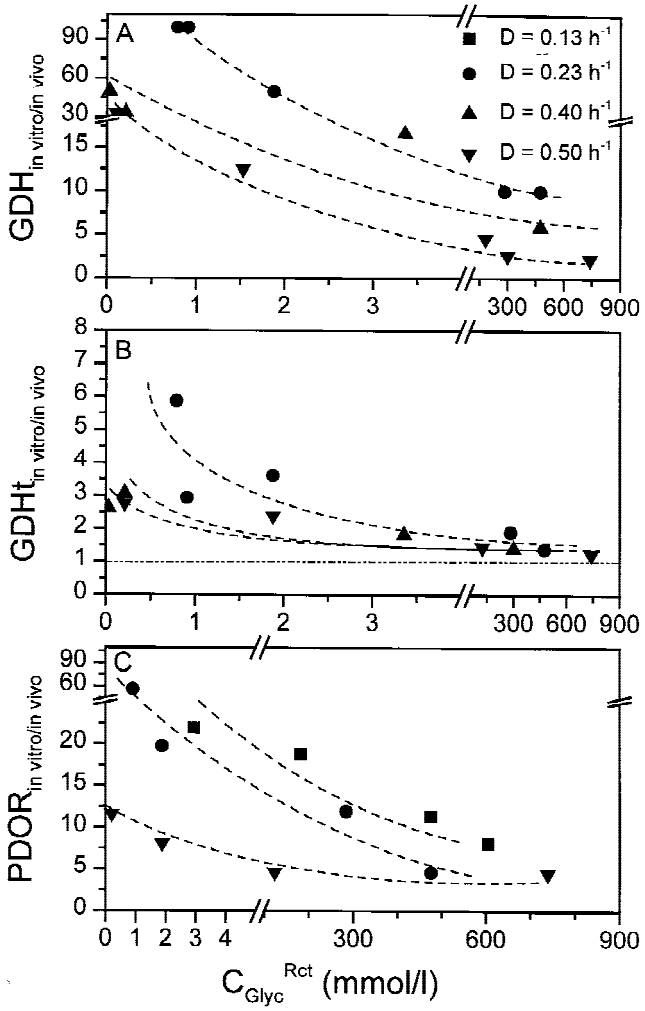

Figure 3. Ratio of in vitro to in vivo activities of (A) glycerol dehydrogenase, (B) glycerol dehydratase, and (C) 1,3-propanediol oxidoreductase as a function of glycerol concentration in culture at different dilution rates.

$GDHt_{spec}$ was somewhat less sensitive to C^{Rct}_{Glyc} compared with GDH_{spec} and $PDOR_{spec}$; that is, it was a strict function of the glycerol uptake rate, as revealed in Figure 4B, sharply declining at low values of q_{Glyc} and reaching a value of about 0.35 U/mg protein at q_{Glyc} greater than about 110 mmol/L, irrespective of the dilution rate. Unlike GDH_{spec} and $GDHt_{spec}$, $PDOR_{spec}$ correlated better with the specific formation rate of 1,3-PD than with q_{Glyc} (Fig. 5B). This may be due to the fact that 1,3-PD is the product of PDOR and exerts feedback inhibition (Johnson and Lin, 1987). It was found that $PDOR_{spec}$ decreased with increasing 1,3-PD concentration at all dilution rates studied (data not shown).

The ratios of in vitro and in vivo activities of GDHt and PDOR (Fig. 3B and C) followed qualitatively similar trends as those for GDH with respect to the effects of glycerol concentration and growth rate. There were, however, some remarkable quantitative differences among them. In general, $GDHt_{in\ vitro/in\ vivo}$ was much lower than $GDH_{in\ vitro/in\ vivo}$ and $PDOR_{in\ vitro/in\ vivo}$, indicating that this enzyme was more effectively utilized. At high glycerol concentrations,

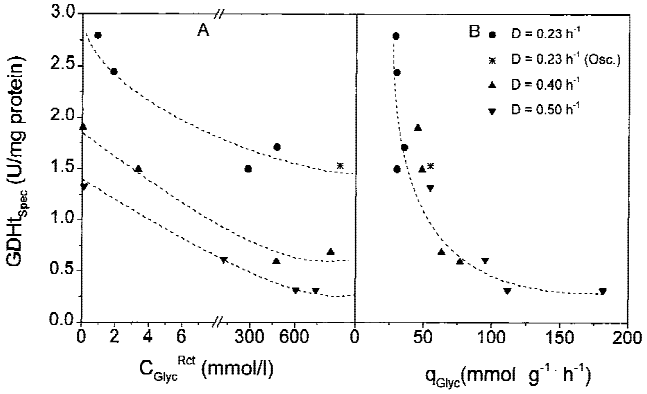

Figure 4. Specific activity of glycerol dehydratase (GDHt) as a function of glycerol concentration in the reactor (C^{Rct}_{Glyc}) (A) and specific glycerol uptake rate (q_{Glyc}) (B) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

$GDHt_{in\ vitro/in\ vivo}$ reached a very low value of 1.2, indicating that the maximum capacity of GDHt was nearly exhausted. The lowest values for $GDH_{in\ vitro/in\ vivo}$ and $PDOR_{in\ vitro/in\ vivo}$ were 2.2 and 4.5, respectively.

Three conclusions can be drawn from the results presented for the enzymes and metabolic fluxes associated with glycerol dissimilation in *K. pneumoniae* under steady-state conditions. First, glycerol concentration in the culture is a key parameter that inversely affects the synthesis of the enzymes GDH, GDHt, and PDOR, but has a positive effect on the utilization of the enzymes synthesized. The responses of these enzymes are completely different from those associated with pyruvate metabolism in this strain under the same conditions (Menzel et al., 1997c). For other microorganisms, such as *E. coli* (Cooney et al., 1981) and *Pseudomonas aeruginosa* (Matin, 1981), the in vitro activities of some enzymes also increased with increased substrate concentration. The mechanisms underlying these differences

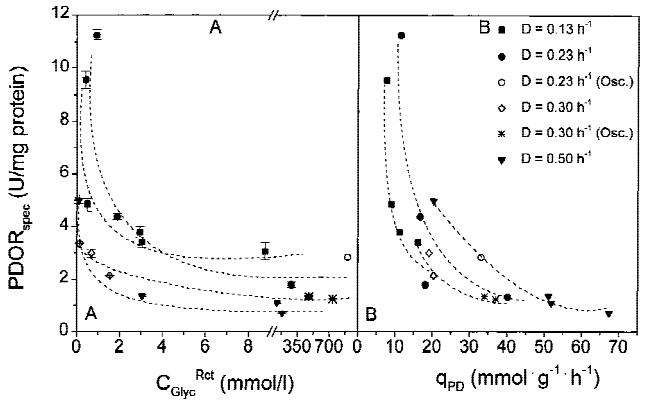

Figure 5. Specific activity of 1,3-propanediol oxidoreductase (PDOR) as a function of glycerol concentration in the reactor (C^{Rct}_{Glyc}) (A) and specific glycerol uptake rate (q_{Glyc}) (B) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

are not clear. Second, whereas the flux through the oxidative pathway of glycerol dissimilation is governed mainly by the regulation of enzyme activity on metabolic levels, the flux through the reductive pathway is largely controlled by the synthesis of enzymes on genetic levels. It is worthy of mention that dihydroxyacetone is an inducer of the *dha* regulon, but has a feedback inhibition on GDH at the same time. This means that this intermediate affects glycerol dissimilation on both genetic and metabolic levels. Obviously, the regulations on these two levels interact with each other, particularly at low glycerol concentrations. To understand these interactions a system and mathematical approach, such as modeling of the metabolic and genetic networks, would be useful. Finally, GDHt is the major rate-limiting enzyme for the dissimilation of glycerol and thus for the formation of 1,3-PD in *K. pneumoniae* at high glycerol concentration. This is in accordance with the findings of Boenigk et al. (1993) and Abbad-Andaloussi et al. (1996) for 1,3-PD formation in *C. freundii* and *C. butyricum*, respectively. It has been argued that the physiological meaning of this limiting step is to avoid the accumulation of 3-hydroxypropionaldehyde (3-HPA), which was found to be very toxic to the growth of another 1,3-PD-producing microorganism *Enterobacter agglomerans* (Barbirato et al., 1996). More experimental investigations are needed to address the possible accumulation of 3-HPA in other 1,3-PD-producing strains.

In Vitro and In Vivo Enzyme Activities Under Sustained Oscillations

The activities of enzymes GDH, GDHt, and PDOR were investigated for cultures undergoing sustained oscillations at two dilution rates, 0.23 and 0.30 h^{-1}. The consumption rate of substrate and formation rates of biomass and different products during the oscillations were exactly the same as those reported by Menzel et al. (1996) and Zeng et al. (1996). The specific activities of GDH, GDHt, and PDOR during an oscillation cycle at $D = 0.23\ h^{-1}$ are depicted in Figure 6. Similar results were obtained for the oscillations at $D = 0.30\ h^{-1}$. GDH_{spec}, $GDHt_{spec}$, and $PDOR_{spec}$ all underwent periodic changes, which had the same frequencies. They followed the same oscillation patterns as CO_2 evolution and growth rate (data not shown). A possible reason for the simultaneous oscillations of these three enzymes may be that their genes belong to one and the same regulon, *dha* (Forage and Lin, 1982; Tong et al., 1991), and thus subject to the same regulations. The magnitudes of the oscillations of these enzymes corresponded to their concentration levels under steady state: GDH displayed a minimum of 2.3 and a maximum of 4.6 U/mg protein; PDOR a minimum of 1.2 and a maximum of 4.3 U/mg protein; and GDHt a minimum of 0.8 and a maximum of 2.0 U/mg protein. It is noticed that the average values of GDH_{spec}, $GDHt_{spec}$, and $PDOR_{spec}$, during an oscillation cycle, agree well with their corresponding steady-state values at the same dilution rates and average glycerol concentrations, as shown in Figures 2, 4, and 5, respectively. These results are consistent with the findings that the average specific rates of glycerol uptake and formation of products deriving either directly from glycerol (1,3-PD) or from the upstream of pyruvate metabolism (e.g., succinic acid) are not significantly affected during oscillation (Zeng et al., 1996). In contrast, a key enzyme of pyruvate metabolism, pyruvate:formate-lyase, was found to change considerably (Menzel et al., 1997c) as did the products originating from pyruvate metabolism (Zeng et al., 1996).

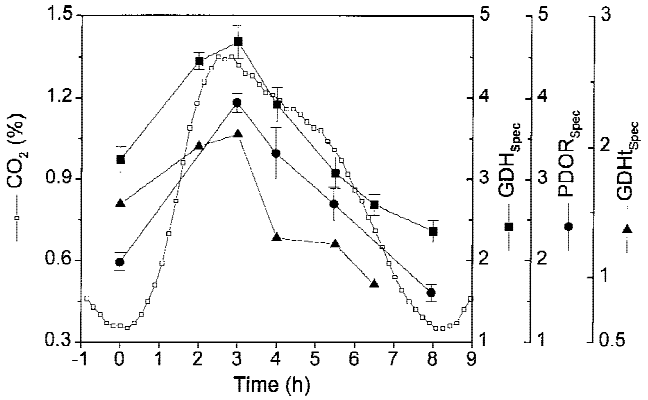

Figure 6. CO_2 concentration in the effluent gas and specific activities (U/mg protein) of enzymes GDH, GDHt, and PDOR during an oscillation cycle in a continuous culture with feed glycerol concentration = 99 g/L and $D = 0.23\ h^{-1}$. Also shown are standard deviations for GDH and PDOR assays. The point of minimum CO_2 evolution was taken as time zero.

Figure 7 reveals that, during oscillations, the ratios of in vitro to in vivo activities of the three enzymes varied. Compared with those for steady states at similar growth rates and glycerol concentrations (large excess) (Fig. 3A–C) the utilization of the enzyme capacity during oscillation was generally lower for GDH and PDOR. The effect on GDHt was not significant. Quantitative information about the concentrations of intracellular metabolites and cofactors under

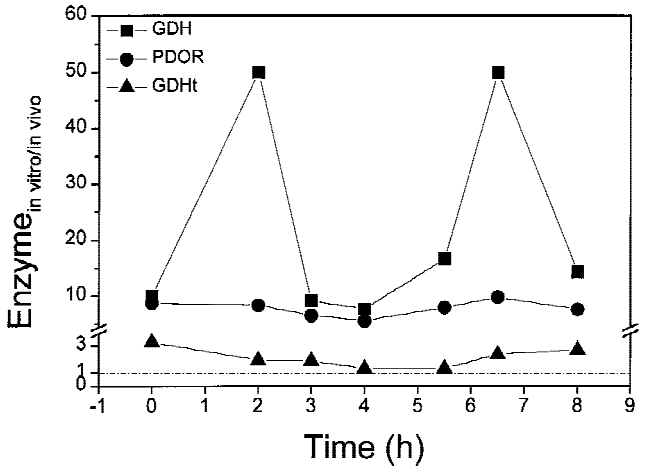

Figure 7. Ratios of in vitro to in vivo activities for enzymes GDH, GDHt, and PDOR during an oscillation cycle in the continuous culture of Figure 6.

conditions of steady state and oscillation is needed to understand these differences. Menzel et al. (1997c) found that the intracellular concentrations and the ratio of $NADH_2$ and NAD^+ changed during oscillations compared with their steady-state values. These changes may directly affect the in vivo activities of at least two of the enzymes, namely GDH and PDOR.

Transient Behavior After a Step Change of Substrate Concentration

The transient behavior of continuous culture after a step change of glycerol concentration in the feed (C^{Med}_{Glyc}) was studied at a dilution rate of $D = 0.30\ h^{-1}$. In one experiment, a steady state was first achieved at $C^{Med}_{Glyc} = 30$ g/L, which was then shifted to 60 g/L, and in another experiment from $C^{Med}_{Glyc} = 10$ to 60 g/L. Figures 8 and 9 show the time courses of substrate, biomass, and product concentrations as well as the specific rates of the main products during the transient phases of the two cultures, respectively. In the experiment with an initial $C^{Med}_{Glyc} = 30$ g/L (Fig. 8), the concentrations of biomass, acetate, and 1,3-PD first increased and then declined as the residual glycerol concentration accumulated in the culture. In contrast, CO_2 evolution rate and ethanol formation declined immediately after the elevation of glycerol concentration. The CO_2 evolution rate reached a minimum value close to zero after about 6 h, indicating an almost complete inhibition of the decarboxylation of pyruvate. The CO_2 evolution gradually recovered and began to oscillate after about 8 h. Surprisingly, the specific formation rates of acetic acid and 1,3-PD kept increasing continuously during this time period, although acetic acid is a main product of the decarboxylation of pyruvate and the formation of 1,3-PD is affected by the reducing equivalents released during the decarboxylation. The response of the second culture followed similar trends but in a more prompt and profound manner. The CO_2 evolution rate reached a minimum value close to zero after only about 2 h and remained at this low level for about 20 h (six volume exchanges) before it recovered. The culture oscillated as well (not shown). This oscillatory behavior, after a large perturbation of the substrate concentration from a low to a high level, is typical for this fermentation process (Menzel et al., 1996). Figure 10 shows that the specific activities

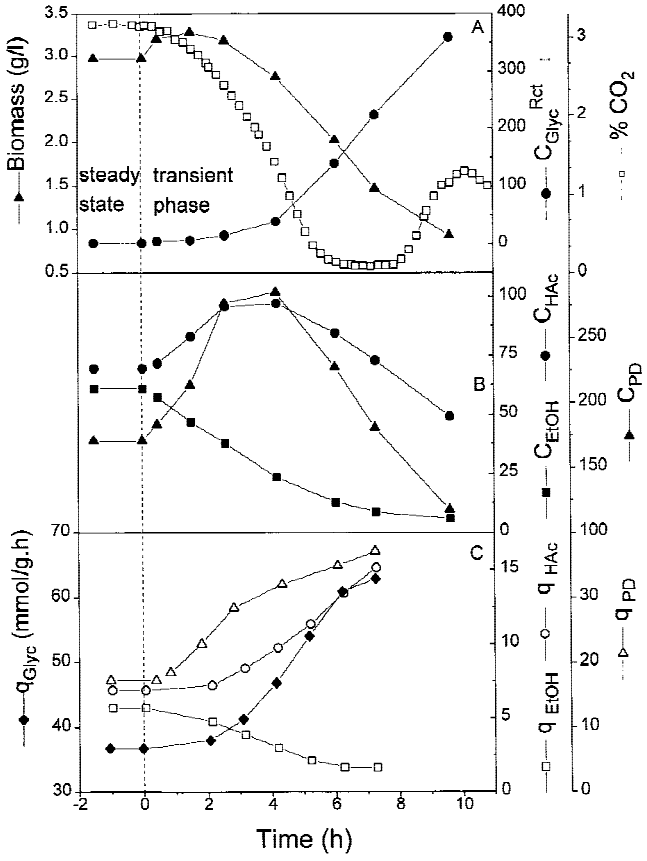

Figure 8. Time courses of (A) concentrations of biomass, substrate (mmol/L), and CO_2 in the effluent gas; (B) concentrations (mmol/L) of products; and (C) specific formation rates ($mmol \cdot g^{-1} \cdot h^{-1}$) of products during transition of a continuous culture after a step change of glycerol feed concentration from 30 to 60 g/L at a dilution rate of $0.30\ h^{-1}$. The time point of substrate shifting was taken as time zero.

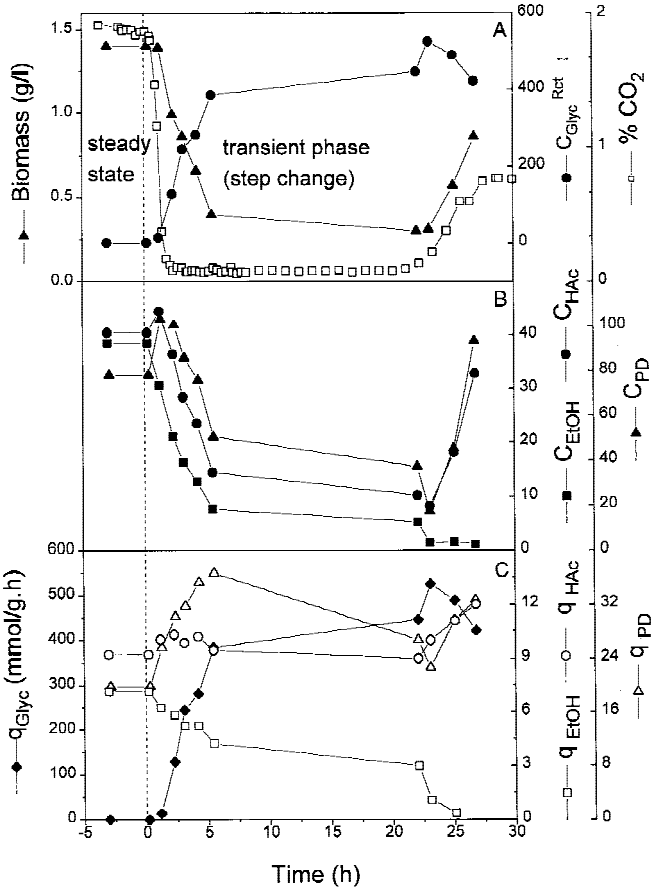

Figure 9. Time courses of (A) concentrations of biomass, substrate (mmol/L), and CO_2 in the effluent gas; (B) concentrations (mmol/L) of products; and (C) specific formation rates (mmol/g · h) of products during transition of a continuous culture after a step change of glycerol feed concentration from 10 to 60 g/L at a dilution rate of $0.30\ h^{-1}$. The time point of substrate shifting was taken as time zero.

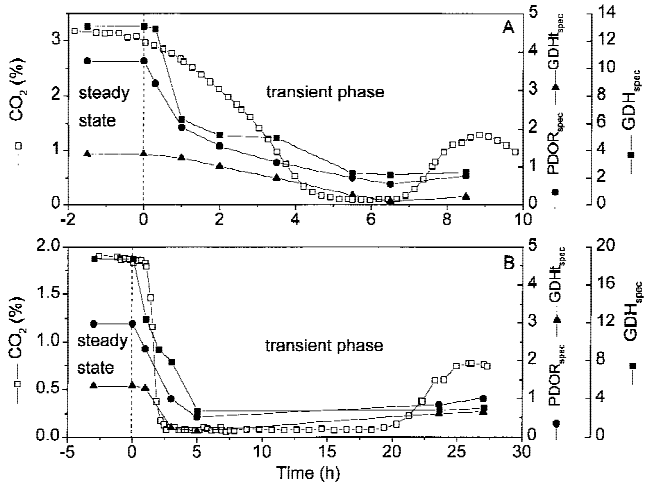

Figure 10. Time courses of the specific activities (U/mg protein) of GDH, GDHt, and PDOR during the transition of continuous cultures: (A) after a step change of glycerol feed concentration from 30 to 60 g/L (cf. Fig. 8); and (B) after a step change of glycerol feed concentration from 10 g/L to 60 g/L (cf. Fig. 9).

of GDH, GDHt, and PDOR also responded immediately to the change of substrate concentration. The decline of GDH and PDOR was more rapid than that of GDHt. As in the case of cell growth and CO_2 evolution the specific activities of the enzymes changed more promptly and profoundly in the second culture with a larger perturbation.

Figure 11 shows the ratios of in vitro/in vivo activities of GDH, GDHt, and PDOR during the transition after a step change from 30 to 60 g/L. It is noticed that the ratios for all the three enzymes first increased, indicating that the immediate responses of the enzymes to an increased substrate concentration were not to increase their utilization as one would expect from the aforementioned results for the steady states. An increased utilization of the enzymes was only observed after about 1 hour. In the second experiment with a step change (Fig. 9) the ratios for the three enzymes decreased immediately after the increase of feed glycerol concentration (data not shown). The results shown in Figures 10

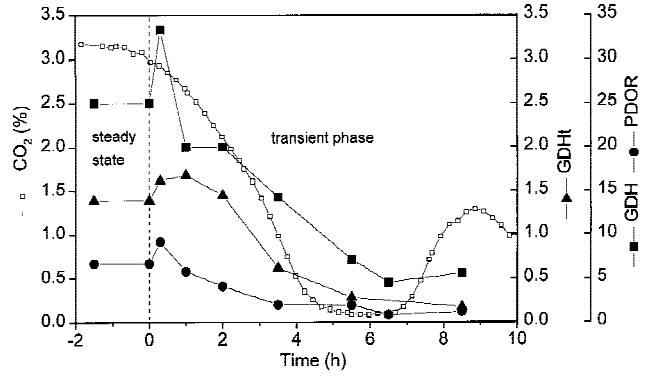

Figure 11. Ratios of in vitro/in vivo activities of GDH, GDHt, and PDOR during the transition of a continuous culture after a step change of glycerol feed concentration from 30 to 60 g/L (cf. Fig. 8).

and 11 demonstrate that the transient responses of growth and product formation, as depicted in Figures 8 and 9, are combined outcomes of regulations of enzyme synthesis on the gene level and enzyme activities on the metabolic level.

Consistent with the experimental results obtained under conditions of both steady state and sustained oscillations these results emphasize that both the genetic and metabolic networks in *K. pneumoniae* anaerobically grown on glycerol are sensitive to changes of substrate concentration in culture. Thus, any attempt to describe mathematically the kinetic and dynamic phenomena reported in this work and those in our previous work (Menzel et al., 1996; Zeng et al., 1996) should take into account regulation and interaction of the genetic and metabolic networks. Unfortunately, our present knowledge of these aspects is generally still very limited. For example, the time constants for the dynamic responses of the genetic and metabolic networks are not known but are desperately needed to describe quantitatively the oscillatory and transient phenomena depicted in Figures 6 to 11. Glycerol metabolism by *K. pneumoniae* may serve as a good example and a model system for improving our quantitative understanding of regulation of metabolic pathways and fluxes. The pathways of glycerol metabolism are now well understood so that the in vitro and in vivo activities of enzymes under different conditions can be measured and compared with one another, as shown in this work. The genes for the dissimilation of glycerol and 1,3-propanediol (*dha* regulon) in *K. pneumoniae* (Cameron et al., 1998; Tong et al., 1991) and *C. freundii* (Daniel and Gottschalk, 1992; Daniel et al., 1995) have been cloned and sequenced. We have recently constructured synthetic *dha* regulons that allow separate or combined overexpression of the enzymes GDHt and PDOR (results not published). The genetic tools should be very useful for studying the regulations and interactions of metabolic pathways and fluxes involved in glycerol fermentation. They are important building blocks for a rational metabolic engineering of microorganisms and deserve further study. In the long term, this may lead to the development of more effective processes to convert glycerol or sugars to the industrially valuable product, 1,3-propanediol.

CONCLUSION

Glycerol concentration in culture is a key parameter that inversely affects the in vitro activities (synthesis) of glycerol dehydrogenase, glycerol dehydratase, and 1,3-propanediol oxidoreductase, but has a positive effect on their in vivo activities under steady-state conditions. In general, the flux through the oxidative pathway of glycerol dissimilation is governed mainly by the regulation of in vivo enzyme activity at the metabolic level, whereas flux through the reductive pathway is largely controlled by the synthesis of enzymes. Glycerol dehydratase is the major rate-limiting enzyme for the consumption of glycerol and thus for the formation of 1,3-PD in *K. pneumoniae* at high glycerol concentrations. Results obtained for cultures under oscillation

and/or after a step change revealed that not only the in vivo activities but also the in vitro activities (synthesis) of the enzymes fluctuate under these dynamic conditions. The regulation and interaction of both the genetic and metabolic networks should be considered when modeling the dynamics of the culture and attempting to manipulate the metabolic fluxes.

References

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

Abbad-Andaloussi, S., Guedon, E., Spiesser, E., Petitdemange, H. 1996. Glycerol dehydratase activity: the limiting step for 1,3-propanediol production by *Clostridium butyricum* DSM 5431. Lett. Appl. Microbiol. **22**: 311–314.

Ahrens, K. 1997. Glyceringärung von *Klebsiella pneumoniae* zu 1,3-Propandiol. Diplomarbeit, M.S. thesis, Technical University of Dresden, Germany.

Barbirato, F., Grivet, J. P., Soucaille, P., Bories, A. 1996. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by Enterobacter species. Appl. Environ. Microbiol. **62**: 1448–1451.

Boenigk, R., Bowien, S., Gottschalk, G. 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii.* Appl. Microbiol. Biotechnol. **38**: 453–457.

Cameron, D. C., Altaras, N. E., Hoffman, M. L., Shaw, A. J. 1998. Metabolic engineering of propanediol pathways. Biotechnol. Prog. **14**: 116–125.

Cooney, C. L., Kopolov, H. M., Häggström, M. 1981. Transient phenomena in continuous culture, pp. 143–168. In: P. H. Calcott (ed.), Continuous culture of cells, vol. 2. CRC Press, Boca Raton, FL.

Daniel, R., Gottschalk, G. 1992. Growth temperature-dependent activity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii* dha regulon. FEMS Microbiol. Lett. **100**: 281–286.

Daniel, R., Boenigk, R., Gottschalk, G. 1995. Purification of 1,3-propaneiol dehydrogenase from *Citrobacter freundii* and cloning, sequencing, and overexpression of the corresponding gene in *Escherichia coli.* J. Bacteriol. **177**: 2151–2156.

Deckwer, W.-D. 1995. Microbial conversion of glycerol to 1,3-propanediol. FEMS Microbiol. Rev. **16**: 143–149.

Forage, R. G., Foster, M. A. 1982. Glycerol fermentation in *Klebsiella pneumoniae:* functions of the coenzyme B12-dependent glycerol and diol dehydratases. J. Bacteriol. **149**: 413–419.

Forage, R. G., Lin, E. C. C. 1982. dha system mediating aerobic and anaerobic dissimilation of glycerol in *Klebsiella pneumoniae* NCIB 418. J. Bacteriol. **151**: 591–599.

Homann, T., Tag, C., Biebl, H., Deckwer, W.-D., Schink, B. 1990. Fermentation of glycerol to 1,3-propanediol by Klebsiella and Citrobacter strains. Appl. Microbiol. Biotechnol. **33**: 121–126.

Honda, S., Toraya, T., Fukui, S. 1980. In situ reactivation of glycerol-inactivated coenzyme-B12-dependent glycerol dehydratase and diol dehydratase. J. Bacteriol. **143**: 1458–1465.

Johnson, T., Lin, E. C. C. 1987. *Klebsiella pneumoniae* 1,3-propanediol:NAD^+ oxidoreductase. J. Bacteriol. **169**: 2050–2054.

Lin, E. C. C. 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. **30**: 535–578.

Lowry, H. O., Rosebrough, J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**: 265–275.

Matin, A. 1981. Regulation of enzyme synthesis as studied in continuous culture, pp. 69–97. In: P. H. Calcott (ed.), Continuous culture of cells, vol. 2. CRC Press, Boca Raton, FL.

Menzel, K., Zeng, A.-P., Biebl, H., Deckwer, W.-D. 1996. Kinetic, dynamic and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. Part I: The phenomena and characterization of oscillation and hysteresis. Biotechnol. Bioeng. **52**: 549–560.

Menzel, K., Zeng, A.-P., Deckwer, W.-D. 1997a. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae.* Enzyme Microbiol. Technol. **20**: 82–86.

Menzel, K., Zeng, A.-P., Deckwer, W.-D. 1997b. Enzymatic evidence for an involvement of pyruvate dehydrogenase in anaerobic glycerol metabolism by *Klebsiella pneumoniae.* J. Biotechnol. **56**: 135–142.

Menzel, K., Ahrens, K., Zeng, A.-P., Deckwer, W.-D. 1997c. Kinetic, dynamic and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. Part IV: Enzymes and fluxes of pyruvate metabolism. Biotechnol. Bioeng. (in press).

McGregor, W. G., Phillips, J., Suelter, C. H. 1974. Purification and kinetic characterization of a monovalent cation-activated glycerol dehydrogenase from *Aerobacter aerogenes.* J. Biol. Chem. **249**: 3132–3139.

Ruch, F. E., Lengeler, J., Lin, E. C. C. 1974. Regulation of glycerol catabolism in *Klebsiella aerogenes.* J. Bacteriol. **119**: 50–56.

Stinson, S. C. 1995. Fine and intermediate chemicals makers emphasize new products and processes. Chem. Eng. News **17**: 10–14.

Skraly, F. 1997. Metabolic engineering of an improved 1,3-propanediol fermentation, Ph.D. thesis, University of Wisconsin–Madison, Madison, WI, USA.

Tong, I.-T., Liao, H., Cameron, D. C. 1991. 1,3-Propanediol production by *Escherichia coli* expressing genes from *Klebsiella pneumoniae* dha regulon genes. Appl. Environ. Microbiol. **57**: 3541–3546.

Toraya, T., Fukui, S. 1977. Immunochemical evidence for the difference between coenzyme-B12-dependent diol dehydratase and glycerol dehydratase. Eur. J. Biochem. **76**: 285–289.

Toraya, T., Shirakashi, T., Kosuga, T., Fukui, S. 1976. Substrate specificity of coenzyme B12-dependent diol dehydrase: glycerol as both a good substrate and a potent inactivator. Biochem. Biophy. Res. Commun. **69**: 475–480.

Yakusheva, M. I., Malahov, A. A., Posnanskaya, A. A., Yakovlev, V. A. 1974. Determination of glycerol dehydratase activity by the coupled enzymic method. Anal. Biochem. **60**: 293–301.

Yamada, K., Tani, Y. 1988. Glycerol dehydrogenase and dihydroxyacetone reductase of a methylothrophic yeast, *Hansenula ofunaensis.* Agric. Biol. Chem. **52**: 711–719.

Zeng, A.-P., Deckwer, W.-D. 1995. A kinetic model for substrate and energy consumption of microbial growth under substrate-sufficient conditions. Biotechnol. Prog. **11**: 71–79.

Zeng, A.-P. 1996. Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum.* Bioproc. Eng. **14**: 169–175.

Zeng, A.-P., Menzel, K., Deckwer, W.-D. 1996. Kinetic, dynamic and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. Part II: Analysis of metabolic fluxes and pathways under oscillation and steady-state conditions. Biotechnol. Bioeng. **52**: 561–571.

Zeng, A.-P., Biebl, H., Deckwer, W.-D. 1997. Microbial conversion of glycerol to 1,3-propanediol — recent progress, pp. 264–279. In: B. C. Saha and J. Woodward (eds.), Fuels and chemicals from biomass. ACS symposium series 666. ACS, Washington, DC.