# Kinetic, Dynamic, and Pathway Studies of Glycerol Metabolism by *Klebsiella pneumoniae* in Anaerobic Continuous Culture: I. The Phenomena and Characterization of Oscillation and Hysteresis

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Oscillation and hysteresis phenomena are observed in the anaerobic continuous fermentation of glycerol by *Klebsiella pneumoniae* in long-term cultivations under a variety of conditions. In this work, the conditions for the occurrence of these phenomena are reported and the patterns of cell growth and metabolism under oscillation are characterized. During an oscillation period, the formation rates of $CO_2$, $H_2$, and formate and the consumption rate of alkali periodically pass values of maxima and minima, the latter being close to zero. The formation of biomass and fermentation products such as 1,3-propanediol, acetate, and ethanol also undergo periodic changes which shift maxima and minima. Sustained oscillation occurs only under conditions of substrate excess within a distinct regime. At pH 7.0, it is only found at dilution rates above 0.15 $h^{-1}$ under the experimental conditions. At lower pH values, oscillations are more likely to happen, even at a relatively low dilution rate and low substrate excess. Whereas the amplitude of oscillations at pH 7.0 depends on both the dilution rate and the residual glycerol concentration ($C_{Glyc}$) the interval of oscillations appears to be only a function of $C_{Glyc}$. An increase of $C_{Glyc}$ in culture damps the oscillation and leads to its disappearance at $C_{Glyc}$ = 1100 to 1200 mmol/L (pH 7.0). The operation mode was also found to be an important parameter in determining the stability and actual state of the culture, resulting in hysteresis under certain conditions, particularly at low pH values. Generally, a large perturbation of cultivation conditions tends to cause oscillation and hysteresis. The results unambiguously demonstrate that the oscillation and hysteresis phenomena shown in this work are bound to genuine metabolic fluctuations of the microorganism. They reveal several differences and new features compared with those reported in the literature and cannot be readily explained by the mechanisms known so far. 
Key words: glycerol fermentation • *Klebsiella pneumoniae* • oscillation • hysteresis • growth and metabolism • substrate excess

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## INTRODUCTION

The bioconversion of glycerol is of interest to industry because of the increasing glycerol surplus on the market and the potential uses of the product 1,3-propanediol. The latter is presently discussed as a monomer for the production of polymers (Witt et al., 1994), and especially as an intermediate for the synthesis of heterocycles. We have studied glycerol termentation in a number of microorganisms in the last few years (Biebl et al., 1992; Homann et al., 1990; Zeng, 1996; Zeng et al., 1993, 1994). The metabolism of glycerol by *K. pneumoniae* is of particular interest due to its flexible regulation of the carbon and reducing equivalent fluxes under different conditions (Zeng et al., 1993). The product yield and productivity achieved with this strain are high compared to other strains (Menzel et al., 1995a; Zeng, 1996). Although safety concerns regarding this microorganism appear to hinder its practical application, the favorable properties of this strain for the glycerol fermentation may still be used by transferring the genes encoding the propanediol pathway to a safer host such as *E. coli* by recombinant technology. Advances in this respect have been reported by Cameron et al. (1995).

Recently, we observed sustained oscillation in the growth and metabolism of *K. pneumoniae* during long-term continuous fermentation of glycerol under anaerobic conditions. Depending on the mode of operation, different culture states could be obtained under nearly the same initial cultivation conditions, resulting in hysteresis of the culture. The patterns of these oscillation and hysteresis phenomena show considerable differences from those reported for several other microorganisms such as *Saccharomyces cerevisiae* (Chen et al., 1990; Grosz and Stephanopoulous, 1990; von Meyenburg, 1973; Porro et al., 1988), *Escherichia coli* (Harrison and Topiwala, 1974), *Clostridium acetobutylicum* (Edwards et al., 1972), and *Zymomonas mobilis* (Bruce et al., 1991; Jöbses et al., 1986). These phenomena are not only of technical relevance but also of scientific interest. By



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studying the instability and dynamics of a bioprocess one may get useful information about the regulation mechanisms and metabolic pathways which would otherwise be inaccessible under steady-state conditions.

This article reports on the characteristics of the oscillatory and hysteresis phenomena observed for the anaerobic fermentation of glycerol by *K. pneumoniae* and the conditions for their occurrence. Emphasis has been put on the effects of substrate concentration, dilution rate, pH value, and operation mode. In an accompanying article the metabolic fluxes and pathways under oscillation are analyzed and compared with steady-state data under similar environmental conditions to find out the possible causes for the peculiar behavior of this culture (Zeng et al., 1996).

## MATERIALS AND METHODS

### Organism

In this study, *Klebsiella pneumoniae* DSM 2026, obtained from the German Collection of Microorganisms and Cell Culture (DSM, Braunschweig, Germany), was used.

### Culture Media

For the preculture, 250-mL shake flasks containing 50 mL of preculture medium were prepared. The medium components were 20 g/L glycerol, 3.4 g/L $K_2HPO_4$, 1.3 g/L $KH_2PO_4$, 2.0 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 1.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7\ H_2O$, 2.0 mg/L $CaCl_2$, 0.14 mg/L $ZnCl_2$, 0.2 mg/L $MnCl_2 \cdot 4\ H_2O$, 0.12 mg/L $H_3BO_3$, 0.4 mg/L $CoCl_2 \cdot 6\ H_2O$, 0.04 mg/L $CuCl_2 \cdot 2\ H_2O$, 0.05 mg/L $NiCl_2 \cdot 6\ H_2O$, and 0.07 mg/L $Na_2MoO_4 \cdot 2\ H_2O$.

The medium for the continuous culture was prepared and sterilized in 35-L reservoirs. The composition of the salt nutrient solution per liter of deionized water was as follows: 0.75 g/L KCl, 1.38 g/L $NaH_2PO_4 \cdot H_2O$, 5.35 g/L $NH_4Cl$, 0.28 g/L $Na_2SO_4$, 0.26 g/L $MgCl_2 \cdot 6\ H_2O$, 0.29 g/L $CaCl_2 \cdot 2\ H_2O$, 0.42 g/L citric acid $\cdot H_2O$, 1.0 g/L yeast extract, 3.4 mg/L $ZnCl_2 \cdot 6\ H_2O$, 27 mg/L $FeCl_2 \cdot 6\ H_2O$, 10 mg/L $MnCl_2 \cdot 4\ H_2O$, 0.85 mg/L $CuCl_2 \cdot 2\ H_2O$, 2.35 mg/L $CoCl_2 \cdot 6\ H_2O$, 0.5 mg/L $H_3BO_3$, and 25 μg/L $Na_2MoO_4 \cdot 2\ H_2O$, 0.1 mL/L Desmophen (antifoam). A solution of 78% glycerol was autoclaved separately and added to the salt nutrient solution according to the glycerol concentration required in the feeding medium.

### Bioreactors and Cultivation Conditions

Two bioreactor systems were used in this study. One reactor system was a 4-L SETRIC fermentor with a working volume of 2 L (Set 4V, Setric Genie Industriel, Toulouse, France), which was maintained at constant level with a weight control system (Philips weight control unit PR 1592). All the cultivations except the one over 800 h at different pH values (Fig. 8) were carried out in this fermentor system. The fermentor was equipped with temperature, pH, and agitation speed control. Temperature and agitation speed were maintained constant for all fermentations at 37°C and 300 rpm. pH 7.0 was controlled by automatic addition of 20% NaOH. The consumption of alkali was recorded by a Sartorius balance. During cultivation, sparging with $N_2$ at a flow rate of 0.4 vvm ensured anaerobic conditions.

The second reactor system was a 1-L double-walled fermentor (BCC, Göttingen, Germany) with a working volume of 0.5 L. The culture volume was controlled by weight using scales and a weight control unit from Sartorius (Göttingen, Germany). The controls of other cultivation conditions were done in similar ways as with the 4 L reactor.

Inocula for the bioreactor were prepared in two steps. The first preculture was grown in one 250-mL shake flask for 24 h at 37°C and was used to inoculate four additional shake flasks which represented the inoculum for the fermentor after incubation (10% of reactor volume). The cultivation strategy was the same for all fermentations. After inoculation, a batch phase of ca. 5 h was followed. At the end of the exponential growth phase a continuous feed of medium was started at a defined dilution rate. At the beginning of the continuous culture a low glycerol concentration in the feed was used that ensured substrate limitation. After at least four volume exchanges, steady-state conditions were normally realized and samples for the off-line measurements were taken. Afterwards one of the following three parameters—i.e., glycerol concentration in the medium, dilution rate, or pH—was stepwise changed.

### Analytical Methods

The biomass concentration was measured as absorbance at 650 nm and as dry weight (grams dry weight per liter) as well. For the dry weight measurement, 10 mL of culture was centrifuged in weighed tubes at 4500 rpm for 45 min. The pellet was washed once and dried for 72 h at 60°C.

The concentrations of the products ethanol, acetate, 1,3-propanediol, 2,3-butanediol, and acetoin were measured with a gas chromatograph (Chrompack 483A; detector: FID; 2-m glass column packed with Chromosorb 101; carrier gas: $N_2$; injector temperature: 250°C; detector temperature: 300°C, column temperature between 150° and 220°C). The computer program "Apex Chromatography Work Station" (Autochrom Inc., POB 207, Milford, MA 01757) was used for integration and evaluation of the data. Glycerol, formate, succinate, and lactate were determined with enzymatic test kits from Boehringer (Mannheim, Germany). Pyruvate was measured enzymatically with a test kit from Sigma Diagnostics (St. Louis, MO).

$CO_2$ and $H_2$ evolution rates were calculated from measurement of the carrier flow gas with a mass flowmeter (Brooks Instrument BV, Veenedal, The Netherlands) and

the $CO_2$ and $H_2$ contents in the effluent gas. $CO_2$ was determined on-line with an infrared carbon dioxide analyzer (Unor 6N, Maihak, Hamburg, Germany) for the 4-L reactor. For the 1-L reactor, $CO_2$ was measured together with $H_2$ by a gas chromatograph (Chromopack 437A; detector: WLD; column: Poraplot Q capillary column, 25 m; carrier gas: $N_2$; injector temperature: 150°C; column temperature: 50°C). This gas chromatograph was also automated for an on-line measurement of $H_2$. Calibrations for the gas measurement were done with gas mixtures ($N_2/CO_2/H_2$) from Messer–Griesheim (Germany).

## RESULTS

### Characterization of Oscillation by On-Line and Off-Line Measurements

Long-term continuous fermentations of glycerol by *K. pneumoniae* were carried out under anaerobic conditions and followed by on-line measurements of the gaseous products $CO_2$ and $H_2$ and the alkali consumption in addition to off-line measurements of the biomass and the dissolved fermentation products. The on-line signal of carbon dioxide concentration in the exit gas responds quickly to the change of culture condition and remains constant under steady states. This applies also to the evolution rate of hydrogen. Under certain cultivation conditions, periodic changes of $CO_2$ and $H_2$ concentrations in the exit gas are observed, as shown typically in Figure 1A for a culture at a dilution rate of 0.20 $h^{-1}$ and pH 7.0, indicating oscillation of the culture. The oscillation of a culture can also be clearly identified on-line by the periodic changes of the alkali consumption rate (data not shown). The changes of $CO_2$ and $H_2$ concentrations in the exit gas are nevertheless more significant and easy to follow. During oscillations, the $CO_2$ and $H_2$ concentrations periodically pass maxima and minima at a relatively constant time interval, the latter being close to zero under certain conditions. This kind of oscillation has been observed for more than 100 h in some experiments (cf. Fig. 4 and 8), indicating a sustained nature of this phenomenon.

Off-line measurements of the biomass revealed that, under oscillation conditions, the biomass concentration and hence the specific growth rate ($\mu$) of cells also undergo periodic changes. Typical results are shown in Figure 1B for a culture at $D = 0.20\ h^{-1}$ and pH 7.0. Whereas the amplitude of biomass oscillation is within the range of 20% to 30% of its absolute value (1.02 to 1.34 g/L) the oscillation of $\mu$ is significant, ranging from as low as 0.1 $h^{-1}$ to values (up to 0.3 $h^{-1}$) higher than the dilution rate. As can be ascertained from Figure 1 the maxima and minima of biomass and $\mu$ do not exactly match those of the gas evolution. However, the time intervals (frequencies) of these oscillations are nearly the same. If we divide one oscillation cycle into two phases according to the gas evolution, i.e., a phase (phase I) with increasing gas evolution and another phase (phase II) with declining gas evolution, it can be seen that $\mu$ generally increases in phase I. It rises further at the beginning of phase II and then levels off, reaching its minimum at the end of phase II. The biomass concentration has its minimum in phase I, but changes mainly in phase II. Overall, phase II lasts much longer than phase I. The shifting between the maxima and minima of biomass and $\mu$ may be attributed to the fact that $\mu$ is a calculated parameter representing the instantaneous growth rate of cells, whereas biomass concentration is a measured parameter representing the average amount of cells resulting from growth and dilution over a relatively long time period (depending on the dilution rate). At a growth rate lower than the dilution rate the bleed of cells out of the reactors is higher than the formation rate and vice versa. It is thus understood that the biomass concentration increases or decreases at a certain pace behind the specific growth rate.

The residual glycerol concentration ($C_{Glyc}$) in the culture oscillates as well (Fig. 1C). $C_{Glyc}$ follows a similar pattern as the gas evolution and $\mu$, having, however, somewhat irregular maxima and minima. This is due to the peculiarities of the metabolic pathways, which are discussed elsewhere (Zeng et al., 1996). The oscillations in cell growth and substrate consumption are also reflected by the formation of dissolved fermentation products, as shown in Figure 2, typically for the culture at $D = 0.20\ h^{-1}$ and pH 7.0. The concentration of formate ($C_{For}$) displays exactly the opposite patterns as the gas evolution rates (Fig. 2A). This is expected because $CO_2$ and $H_2$ are mainly released by the cleavage of formate. In contrast, the concentration of ethanol ($C_{EtOH}$) changes in nearly the same way as the evolution rates of $CO_2$ and $H_2$. The changes of the concentrations of acetic acid and 1,3-propanediol have similar patterns, but are not as regular as those for $CO_2$ and biomass. The minor products, lactic acid, succinic acid, pyruvate, and 2,3-butanediol, all oscillate but have more irregular amplitudes and frequencies (Fig. 3). These results indicate that a synchronous metabolism resulting from cell cycle cannot be the prevailing mechanism as suggested for the oscillation of yeast cultures. In fact, the reasons for the changes of oscillation patterns of the different products are not always clear. To gain more insight and to understand the oscillatory phenomena presented above, a consideration of the specific rates of cellular metabolism appears to be more appropriate. This will be addressed in more detail elsewhere (Zeng et al., 1996). The purpose of this article is to characterize the behavior of cultures under oscillation and to identify environmental conditions causing the peculiarities of this fermentation process.

From the results presented above it is clear that both the on-line and off-line measurements can be used to characterize the oscillatory phenomenon encountered in the culture under investigation. The periodic changes of cell growth and the production of main fermentation products during oscillations correlate quite well with the evolution rates of $CO_2$ and $H_2$ which reflect the instantaneous meta-

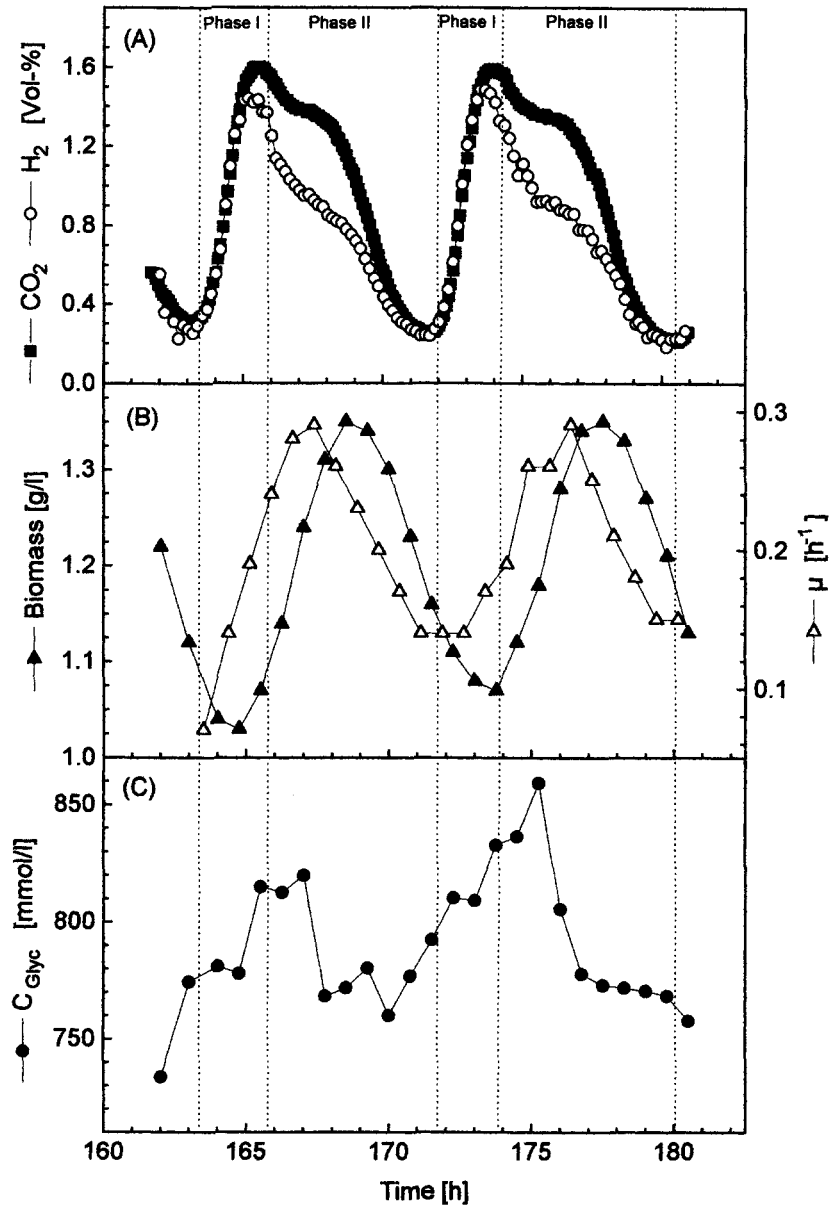


**Figure 1.** Oscillation patterns of formation and/or consumption of $CO_2$ and $H_2$ (A), biomass (B), and glycerol (C) at a dilution rate of 0.20 $h^{-1}$ and medium glycerol concentration of 1087 mmol/L.

bolic activities of the culture and are easily followed by automatic on-line measurements. Therefore, in the following, the on-line measurement of $CO_2$ is used as an on-line parameter for studying the influences of culture conditions and operation mode on the stability of this fermentation process.

## Effects of Dilution Rate and Substrate Concentration at a Neutral pH

The effects of dilution rate and substrate concentration were studied in a series of continuous cultivation at six dilution rates ($D$ = 0.10, 0.15, 0.20, 0.25, 0.35, and 0.45 $h^{-1}$) at pH 7. At each dilution rate, the cultivation was begun with a relatively low substrate concentration ensuring substrate-limited growth. After a steady state had been achieved, the substrate concentration in the medium was subsequently stepwise increased, which ultimately resulted in substrate excess. Under excess substrate conditions cell growth is inhibited by product accumulation (Zeng et al., 1994). The step increase of substrate concentration was normally higher than 200 (about 200 to 500) mmol/L if not mentioned otherwise. No oscillation of the culture could be detected at dilution rates lower than 0.20 $h^{-1}$ with the operation mode applied, even for cultures with residual glycerol concentration as high as 1000 mmol/L (Menzel, 1995b). Clear oscillations were only observed at $D \geq 0.20$ $h^{-1}$ (cf. Figs. 1 and 2.

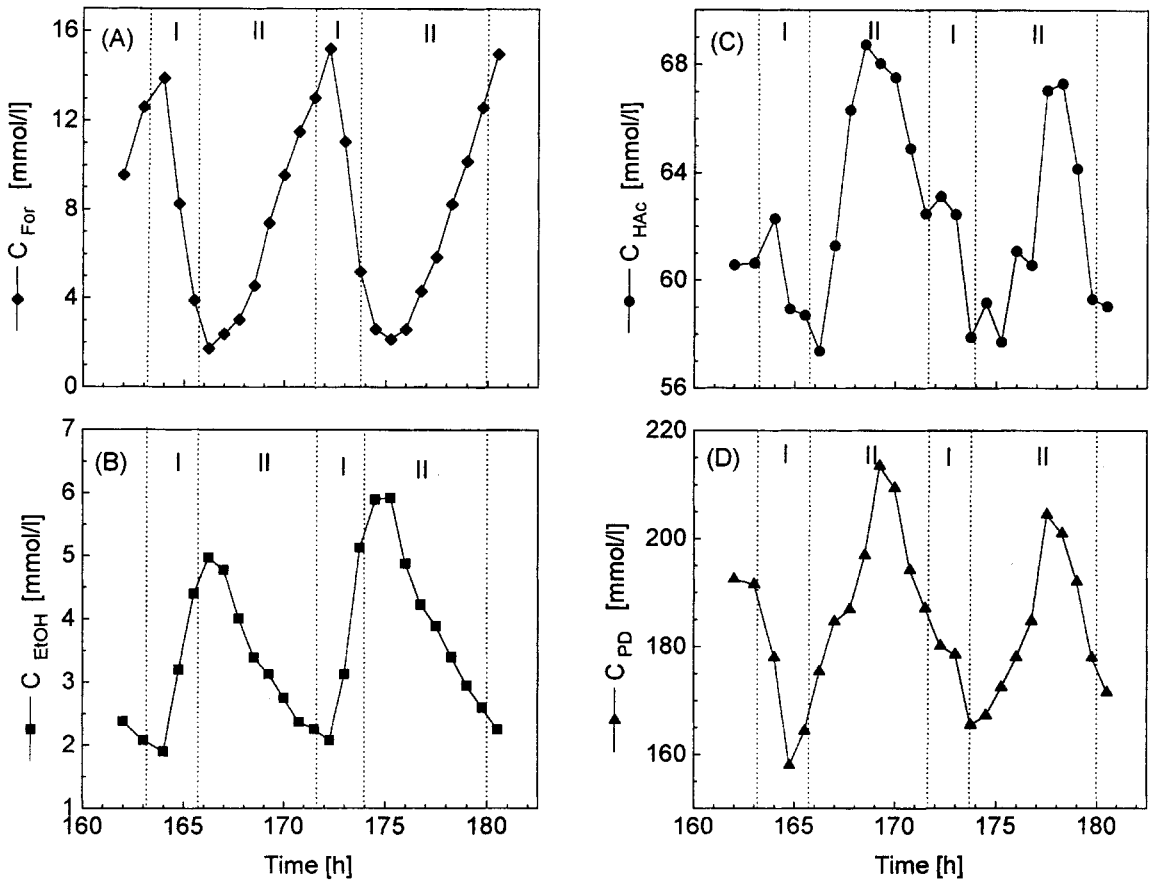


**Figure 2.** Oscillation patterns of concentrations of formate (A), ethanol (B), acetic acid (C), and 1,3-propanediol (D) at a dilution rate of 0.2 $h^{-1}$ and medium glycerol concentration of 1087 mmol/L.

Another prerequisite for the occurrence of oscillation is the condition of an obvious substrate excess. The concentration of residual substrate significantly affects the extent of oscillations as shown in Figure 4 for a culture at $D$ = 0.35 $h^{-1}$. In this culture, sustained oscillation was observed for about 100 h with residual substrate concentrations in the range of about 500 to 750 mmol/L (730 to 950 mmol/L in the feeding medium). Increasing substrate concentration was found to damp the oscillation, resulting in a declined amplitude of oscillation and a lengthened time of oscillation cycles. At a residual substrate concentration of 1196 mmol/L the oscillation disappeared. Surprisingly, the resulting $CO_2$ concentration (1.2%) in the exit gas was very close to the average value under oscillation. Similar results were obtained at a dilution rate of 0.45 $h^{-1}$.

Figures 5 and 6 show the oscillation amplitude of $CO_2$ and the time interval of oscillation cycles as functions of residual substrate concentration in the culture at different dilution rates. The values of amplitude and time interval of oscillations are calculated average values with the last two oscillation cycles at given values of substrate concentration and dilution rate. For all dilution rates studied, the $CO_2$ amplitude decreases with increasing residual substrate concentration (Fig. 5). A nearly linear correlation could be obtained for each dilution rate. All the lines intercept with the $x$-axis (with zero $CO_2$ amplitude) at a residual substrate concentration of about 1200 mmol/L. This implies that the oscillation disappears at the same level of residual substrate concentration, irrespective of the dilution rate. Correspondingly, the time interval of oscillations at all the dilution rates increases with the residual substrate concentration (Fig. 6). The increase is sharper at residual substrate concentrations higher than 700 mmol/L. It is obvious that, at a neutral pH, oscillations occur only within a distinct range of cultivation conditions. In subsequent experiments, it is found that the occurrence of oscillation is not only bound to the dilution rate and substrate excess; rather, the mode of change of cultivation conditions also turns out to be an important factor in determining the culture stability, resulting in the hysteresis phenomenon. This is addressed in what follows.

## Effect of Operation Mode and Hysteresis Phenomenon

The influences of operation mode on the culture stability are demonstrated in Figure 7 for two continuous cultivations carried out at the same dilution rate (0.35 $h^{-1}$) and similar range of substrate concentrations in medium. The difference

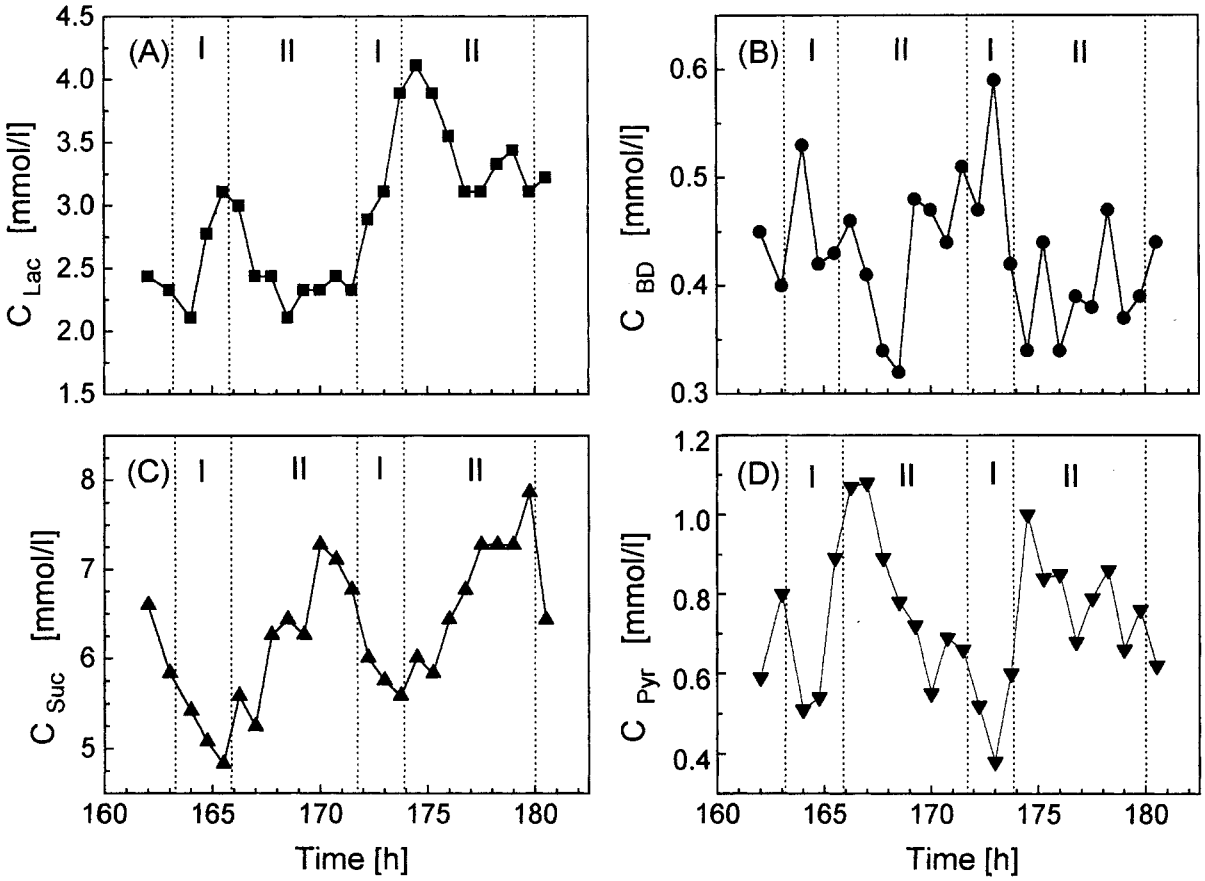


**Figure 3.** Oscillation patterns of concentrations of lactic acid (A), 2,3-butanediol (B), succinic acid (C), and pyruvate (D) at a dilution rate of 0.2 $h^{-1}$ and medium glycerol concentration of 1087 mmol/L.

between culture I and culture II is the way they were started up (concentration shift). Culture I is the one showing oscillation within a medium concentration range of 650 to 1200 mmol/L as visualized by the periodic changes of $CO_2$ in Figure 4. The glycerol consumption given in Figure 7 for these unstable states is the difference between glycerol concentration in the feed medium ($C_{Gly}^{Medium}$) and average residual glycerol concentration in the reactor over at least one oscillation cycle. The transition of culture I from steady state (at $C_{Gly}^{Medium}$ = 379 mmol/L) to oscillation was triggered by a step increase of $C_{Gly}^{Medium}$ from 379 to 729 mmol/L. In a comparative run (culture II), the continuous cultivation was started with $C_{Gly}^{Medium}$ = 223 mmol/L. After the culture reached steady state, $C_{Gly}^{Medium}$ was first increased to 443.5 mmol/L. This resulted in a detectable residual concentration (1.96 mmol/L) well above the substrate concentration for a substrate-limited growth ($K_S$ = 0.26 mmol/L according to Zeng et al. [1994]). Surprisingly, a steady state could be obtained under these conditions. Further increases of $C_{Gly}^{Medium}$ from 443.5 to 706.5 mmol/L and from 706.5 to 869.6 mmol/L all resulted in steady states with relatively high residual substrate concentrations. Substrate consumption and formations of biomass and $CO_2$ under steady states are significantly higher than those under oscillation in a similar range of $C_{Gly}^{Medium}$ (ca. 435 to 1087 mmol/L). At a very high substrate concentration (above about 1000 mmol/

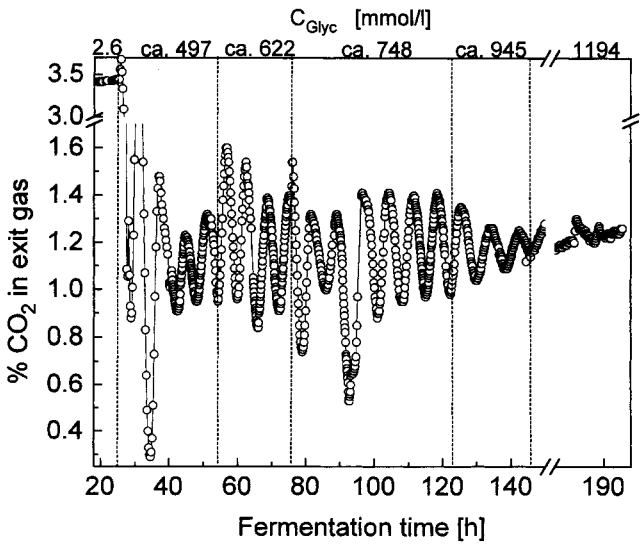


**Figure 4.** Occurrence and disappearance of oscillation at a dilution rate of 0.35 $h^{-1}$ as function of residual glycerol concentration in a reactor ($C_{Glyc}$).

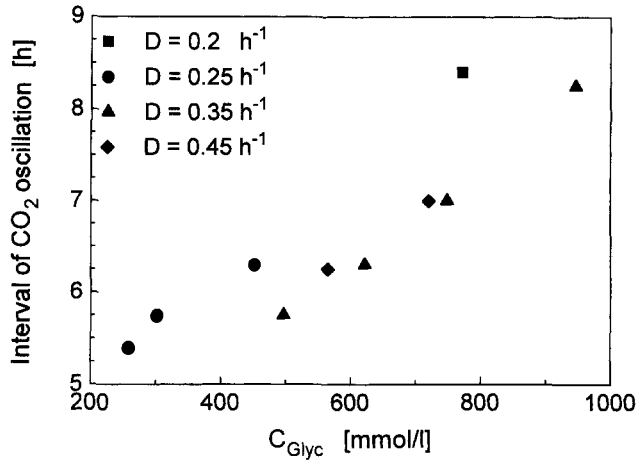


**Figure 5.** Relationship between amplitude of $CO_2$ oscillation in exit gas and residual glycerol concentration in a reactor at different dilution rates.

L), the substrate consumption and formations of biomass and $CO_2$ in culture II are expected to decrease significantly as found for the steady-state continuous culture of *K. pneumoniae* at several dilution rates (Menzel et al., 1995a). The substrate consumption and formations of biomass and $CO_2$ in both culture I and culture II at high values of $C_{Gly}^{Medium}$ (e.g., 1395 mmol/L) would be then very close to each other. If the steady-state results of the two cultures at high $C_{Gly}^{Medium}$ are connected (indicated by the dashed lines in Fig. 7) they very much resemble the Hopf bifurcation behavior of some chemical reactions (Gray and Scott, 1994). It is worth noting that the route with lower metabolic activities instead of the one with higher activities in Figure 7 represents the unstable stationary states (oscillations). It is not clear if the unstable states are unique. In fact, the results presented suggest that the operation state of the *K. pneumoniae* culture under conditions of substrate excess depends not only on the given cultivation conditions but also on the mode of operation (i.e., the magnitude of change from one operation condition to another), implying a kind of multiplicity.

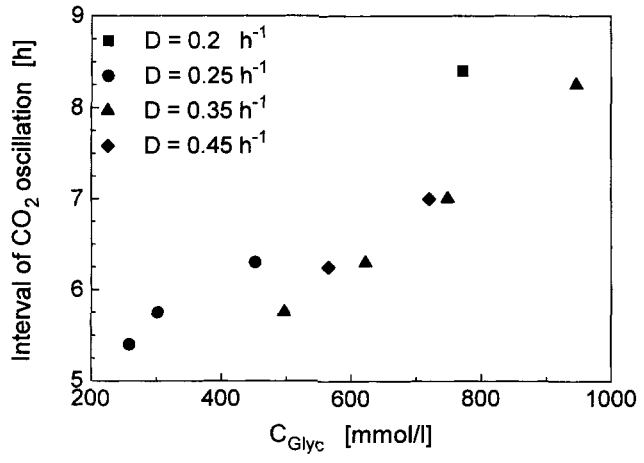


**Figure 6.** Relationship between interval of $CO_2$ oscillation in exit gas and residual glycerol concentration in a reactor at different dilution rates.

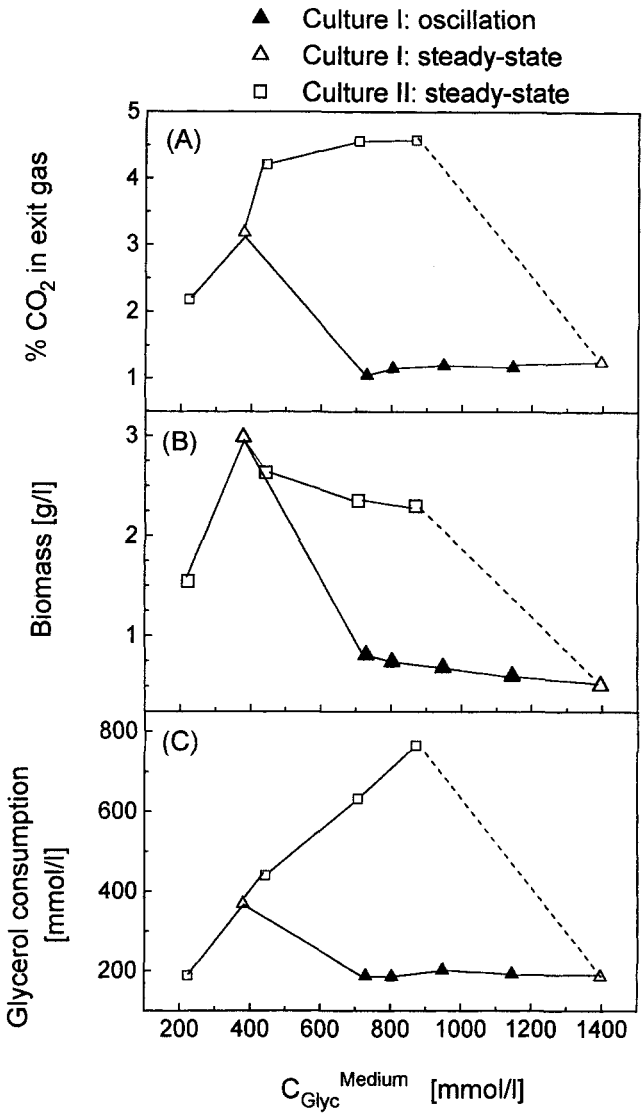


**Figure 7.** Hysteresis phenomena of $CO_2$ in exit gas (A), biomass concentration (B), and glycerol consumption (C) caused by the operation mode (change of substrate concentration) in two cultures with the same dilution rate (0.35 $h^{-1}$) and glycerol concentrations in medium.

## Effects of pH

The effect of pH was studied in experimental runs carried out at relatively constant $C_{Gly}^{Medium}$ (650 mmol/L) but different pH values and dilution rates. In one experiment (Fig. 8), the dilution rate was maintained between 0.22 and 0.23 $h^{-1}$, whereas the pH value was changed stepwise from 7.5 to 6.5 and then up to 7.0 again for the first 150 h of cultivation. At pH values between 7.1 and 7.5 glycerol was completely consumed and steady states were obtained. A decrease of pH from 7.1 to 6.5 led to sustained oscillation for nearly 100 h, accompanied by a high amount of residual glycerol concentration. Under these conditions, an increase of the pH to a neutral level (7.0) did not help to eliminate the oscillation, again demonstrating hysteresis behavior of the culture. As shown in Figure 8, a sharp decline in the dilution rate from

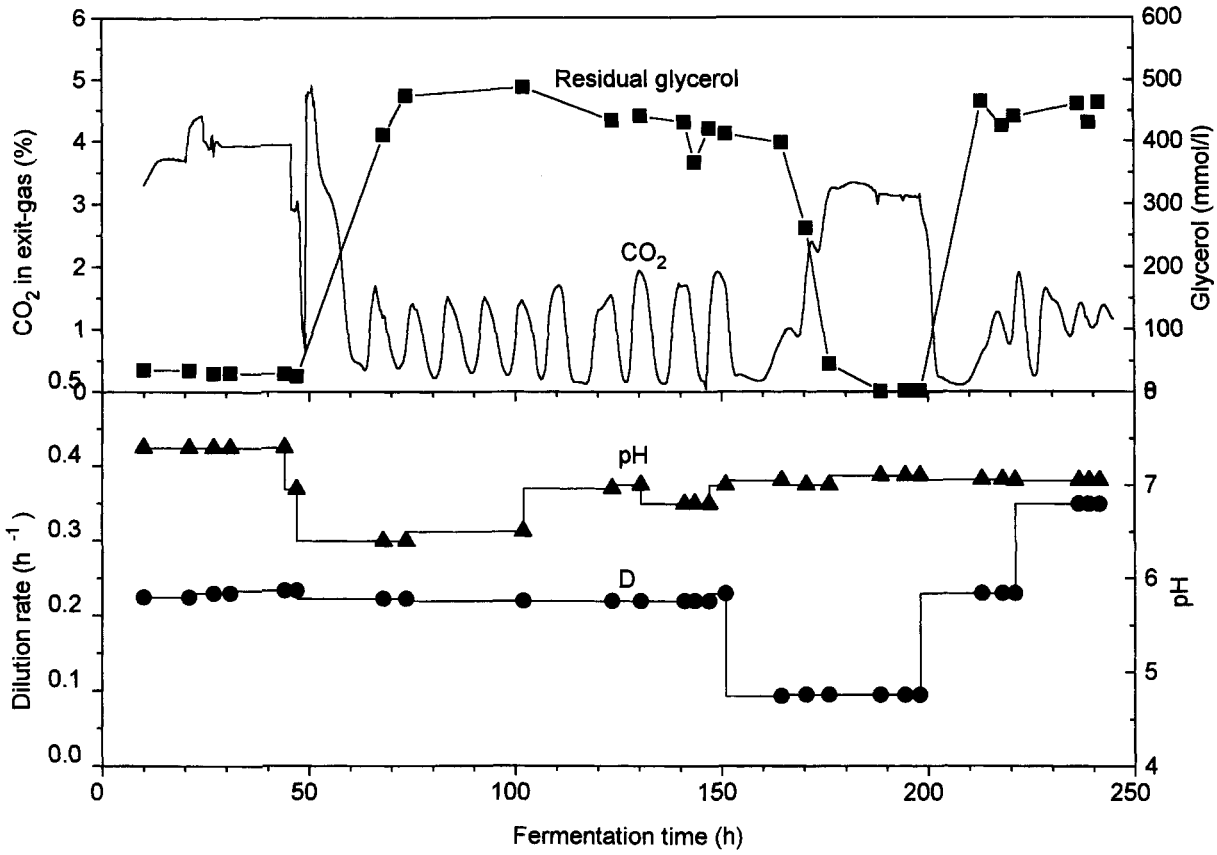


**Figure 8.** Results of a continuous culture of *K. pneumoniae* at different pH values and dilution rates (650 mmol/L glycerol in medium).

0.22 to 0.095 $h^{-1}$ can lead to the disappearance of the oscillation. A return of the dilution rate to 0.22 $h^{-1}$ again caused oscillation. These results reveal that the culture stability is strongly affected by the pH value. The results obtained at neutral pH values in this experiment are also in agreement with the aforementioned findings that oscillation normally occurs at a dilution rate higher than 0.15 $h^{-1}$.

To assess the pH effect over broader range and in more detail, particularly with respect to the behavior of cultures grown under the critical dilution rate as found for oscillation at neutral pH, a long-term cultivation was carried out in a smaller fermentor at a relatively constant low dilution rate (0.09 to 0.1 $h^{-1}$) over a pH range of 5.45 to 7.15 (Fig. 9). The glycerol concentration in medium was also relatively constant (around 600 mmol/L) over the whole period of cultivation. The pH value was first changed downwards from 7.15 to 5.45, and then upward from 5.45 to 7.0. As evidenced by the $CO_2$ production, the residual glycerol concentration and the optical density of the culture at pH values above 6.15 was stable, irrespective of the patterns of pH changes. This is obviously due to the fact that growth under these conditions was under substrate limitation. As pointed out above, an excess of residual substrate in the culture is a prerequisite for oscillation. At pH values below 6.15, culture instability can be inferred by changes of $CO_2$ production and optical density. The culture instability coincides with the occurrence of detectable residual glycerol. A relatively high residual glycerol concentration was observed for the pH range 5.45 to 6.1, which is often an indication of culture instability. The results of Figure 9 clearly demonstrate that there exists no intrinsic critical dilution rate for the occurrence of oscillation and that cultures at low pH values are more inclined to oscillate. It should be mentioned that the $CO_2$ was measured off-line by gas chromatography for this experimental run. Due to the relatively long time interval of sample measurement an exact monitoring of possible periodic changes of $CO_2$ was not possible. Furthermore, the $CO_2$ production is expressed here as an apparent concentration ($C_{CO_2}$), which is calculated by assuming that all $CO_2$ released by cells was dissolved in the culture. $C_{CO_2}$, as a converted value, represents the real amount of production and is therefore convenient for comparing $CO_2$ production at varying pH values and dilution rates because dissolved and dissociated $CO_2$ that is otherwise removed from the reactor by culture bleed can be better accounted for. The calculation of dissolved and dissociated $CO_2$ is done as suggested by Zeng (1995).

Figure 10A shows a comparison of $CO_2$ production calculated in this way as a function of pH. Average values are used for cultures under non-steady state. It was interesting to find that, although $C_{CO_2}$ is essentially constant for pH $\geq$ 6.1 irrespective of the operation mode, it depends strongly on the chronology of pH change at lower pH values. During the downward change of pH, i.e., from steady-state to un-

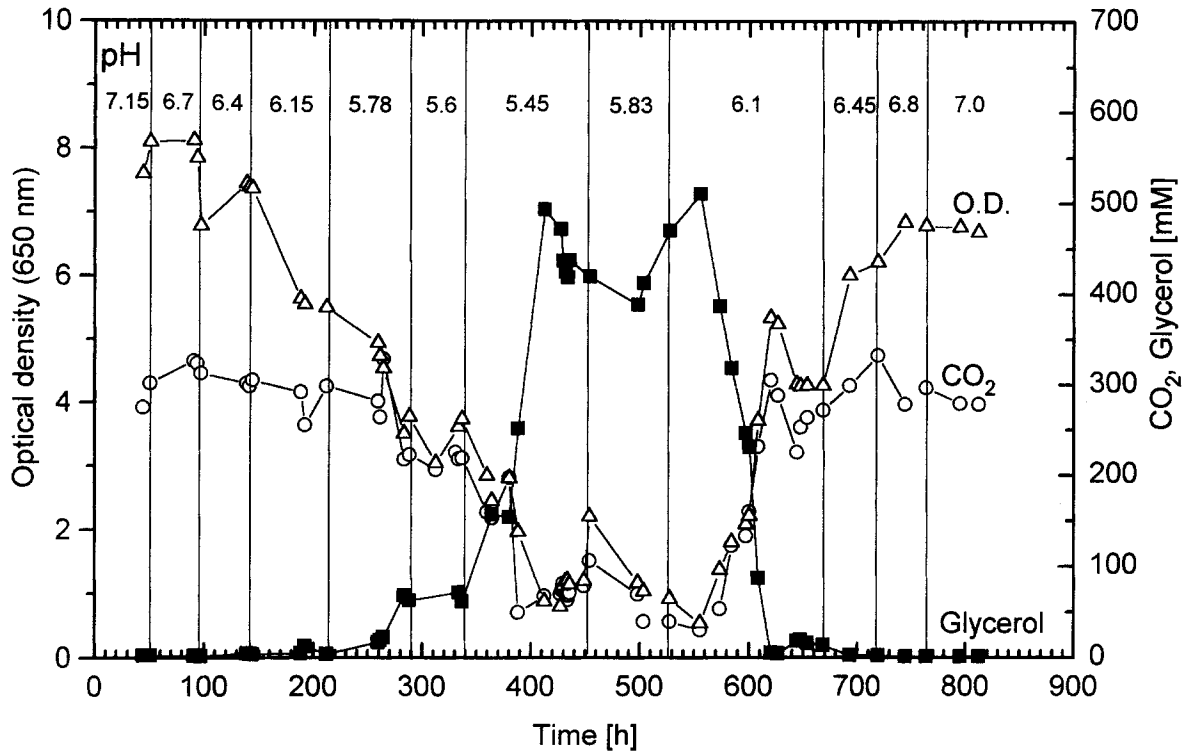


**Figure 9.** Results of a continuous culture of *K. pneumoniae* at different pH values at a constant dilution rate (0.09 to 0.1 $h^{-1}$) and glycerol concentration in medium (about 600 mmol/L).

stable (oscillating) state, much higher production of $CO_2$ was obtained than during the upward change of pH, i.e., upward from an unstable (oscillating) state. Similar results were obtained with glycerol consumption and biomass concentration (Fig. 10B). In the latter case, a slight difference was also observed for pH values $\geq$ 6.1. Generally, a lower biomass concentration was obtained for the cultures during upward changes of pH, although the substrate consumption was nearly the same under these conditions. The formation of the main fermentation products, 1,3-propanediol, acetic acid, and ethanol, showed similar patterns as those of biomass (Fig. 10C and D; data for ethanol not shown). The results of Figure 10 reveal that hysteresis is more likely to happen at pH values below 6.0. It should be pointed out that, in considering the pH effect, possible influences of dilution rate and substrate concentration (particularly the residual substrate in culture) should not be overlooked.

## DISCUSSION

We have reported on the phenomena of oscillation and hysteresis observed in the anaerobic continuous fermentation of glycerol by *K. pneumoniae* and identified the conditions for their occurrence. The results from two reactor systems under a variety of experimental conditions unambiguously demonstrated that these phenomena are bound to genuine metabolic fluctuations of the microorganism under certain culture conditions and are not due to equipment artifacts such as poor regulation of culture conditions, i.e., temperature and pH. Sustained oscillation could be observed over 100 h which can be eliminated and reinduced by changing culture conditions (cf. Figs. 4 and 8). It was found that the culture is very sensitive to pH change. Oscillation and hysteresis are particularly likely to happen at low pH values. However, the appearance of oscillation appears to be bound to substrate excess. No oscillation could be detected under conditions of substrate limitation, irrespective of the pH and other parameters. In contrast, hysteresis of biomass and product formation could be observed under both substrate limitation and substrate excess, as shown in Figures 9 and 10. In this regard, it is worth pointing out that, at very high substrate excess, oscillations (and probably hysteresis) can be diminished, as shown in Figures 4 and 7 for a culture at a neutral pH. This may be due to the strong inhibition by glycerol at high concentration. Edwards et al. (1972) showed that feedback control of substrate concentration improves the stability of continuous cultures growing on inhibiting substrates. Thus, oscillations and hysteresis occur only in a distinct range of operation conditions. At a neutral pH a dilution rate higher than 0.15 $h^{-1}$ seems also to be a prerequisite. Moreover, it was found that the mode of changes of culture conditions is also an important factor affecting the stability of the culture. Generally, a large step change of cultivation conditions, such as substrate concentration, dilution rate, and pH, may lead to sustained oscillation. It may be possible that the perturbations of substrate concentration applied in this work are not high enough to cause oscillation at $D \leq 0.2$ $h^{-1}$ (pH 7.0). An intrinsic

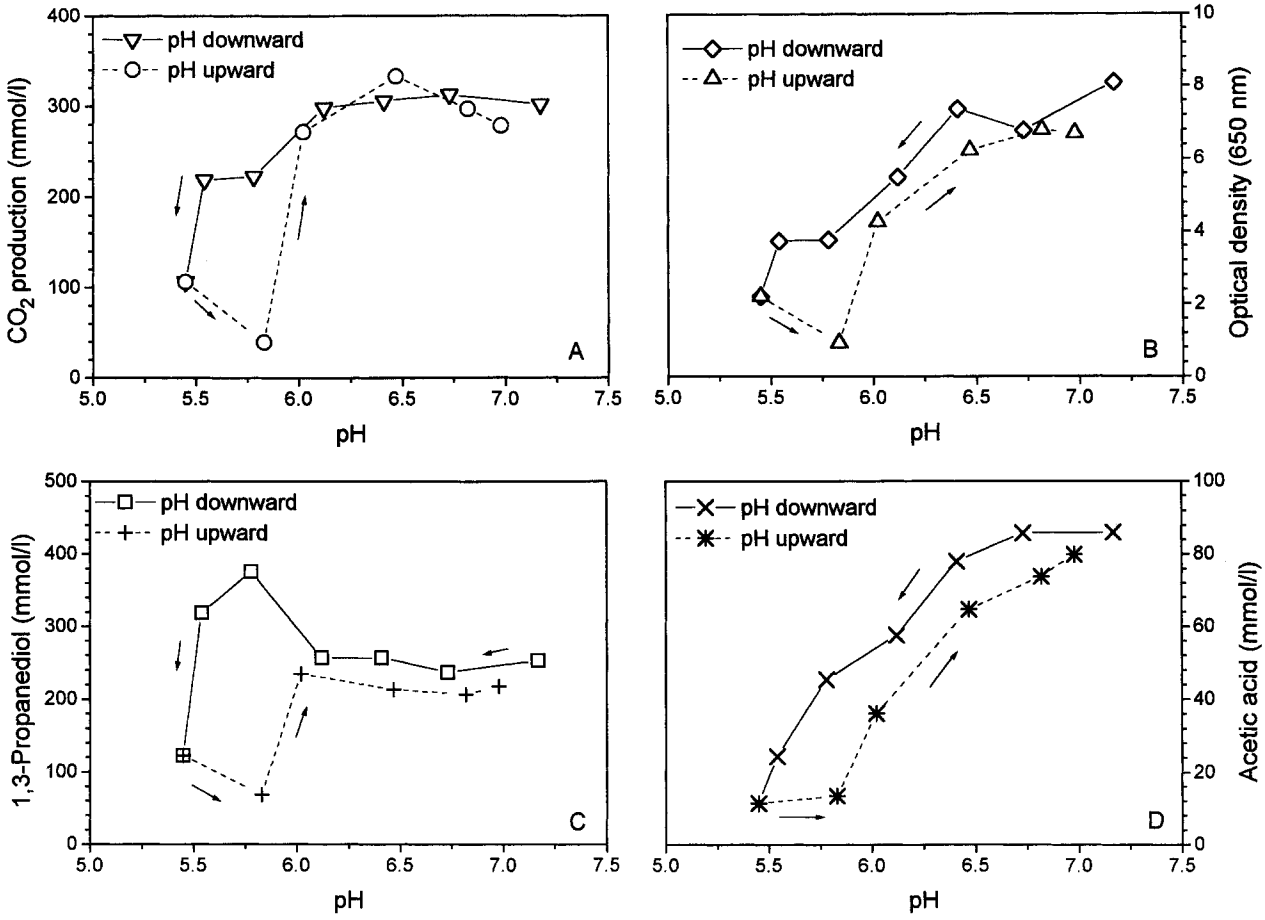


**Figure 10.** Hysteresis phenomena of $CO_2$ in exit gas (A), biomass (B), 1,3-propanediol (C), and acetic acid concentration (D) as functions of pH and operation mode at constant dilution rate (0.09 to 0.1 $h^{-1}$) and glycerol concentration in medium (about 600 mmol/L).

critical dilution rate (or growth rate) for oscillation seems not to exist, as suggested by the experimental results at low pH (cf. Fig. 9).

These findings have practical implications for process development and operation. As discussed elsewhere (Menzel et al., 1995a; Zeng et al., 1993), for an optimal production of 1,3-propanediol in continuous culture it is necessary to run the fermentation under substrate excess at dilution rates between 0.1 and 0.25 $h^{-1}$ (pH 7.0). This is within the regime in which an oscillation of the culture could occur. Because the substrate consumption and product formation under oscillations are much lower compared with those under steady states oscillations should be avoided. This requires careful control of the operating conditions so as to avoid large perturbations of substrate concentration, dilution rate, pH, etc. Low pH values should be avoided as well. From a viewpoint of process control this work demonstrates that the on-line measurements of $CO_2$ and $H_2$ concentration in the exit gas are useful tools to monitor the culture stability. The consumption rate of alkali can also be used as an on-line signal and it is easy and inexpensive to implement. Once periodic changes in these parameters are observed measures should be taken to eliminate them, such as by decreasing the dilution rate and substrate concentration and/or increasing the pH value. The desired operation point could then be achieved by slow adjustment of the parameters.

The phenomena and findings presented in this work are not only of technical relevance but also of scientific interest. In fact, the patterns of oscillation and hysteresis discussed show differences and new features compared to those reported in the literature. Harrison and Topiwala (1974) gave a comprehensive review on the oscillatory phenomena of continuous culture. More recent reviews and discussions of oscillations associated with bacterial and yeast cultures may be found in the works of Clarke et al. (1988), Bellgardt (1994), and Hjortso and Nielsen (1994). Oscillations in microbial cultures generally originate from: (1) feedback between cells and environmental parameters, such as an extracellularly produced metabolite, which may inhibit growth or repress certain enzymes (pattern I); (2) intracellular feedback regulation, such as by an oscillation of intracellular $NADH_2$ or by allosteric control of enzyme activity (pattern II); (3) synchronous growth (cell cycle) of cells such as in baker's yeast (pattern III); and (4) dynamics of cell population such as in mixed cultures (pattern IV). The oscillation

of our culture may principally be fit into pattern I as it is induced by the change of environmental parameters, such as substrate concentration and pH. However, no definable environmental parameters can be identified as being responsible. Growth inhibition by extracellular products such as 1,3-propanediol and ethanol may be involved because all of the main products are inhibitory. However, they cannot be the intrinsic reason(s) since the concentration, and hence the inhibitory effect of products, is usually much higher under steady state than under oscillation. The oscillation can also not be attributed alone to the inhibition by substrate because it is damped by high substrate concentration instead of being enhanced (cf. Fig. 4).

It seems that the real reason(s) should be sought in the intracellular metabolism (pattern II). However, as pointed out by Harrison and Topiwala (1974), oscillating intracellular parameters would only be detectable if a high degree of synchrony existed between the oscillations within the individual cells. For this to happen the oscillating systems in the individual cells must be able to interact through a common environmental parameter. Again, no such a common environmental parameter could be identified so far. In fact, the shifting of maxima and minima and irregular fluctuations of substrate consumption and product formation as shown in Figures 4 and 6 do not suggest the existence of synchronous metabolism. Furthermore, intracellular feedback regulation normally renders a response on a much shorter time-scale, i.e., minutes rather than hours as observed in this culture. In addition to the irregular fluctuations of some products both the amplitude and interval of oscillation (indicated by the $CO_2$ evolution rate) were found to depend mainly on the residual substrate concentration and less on the dilution rate or doubling time (Figs. 5 and 6). It can be thus assumed that the cell cycle cannot be the intrinsic reason either. The results found for synchronous growth (cell cycle) of yeast cultures (pattern III) cannot be directly applied to our system. Clarke et al. (1988) attributed the oscillation phenomena in a continuous culture of *Clostridium acetobutylicum* to the coexistence and periodic shift of acid- and solvent-producing populations (pattern IV). In this kind of culture, oscillations are damped by increasing the dilution rate. However, in our culture, oscillations are damped by decreasing the dilution rate. The coexistence of two distinguished subpopulations in the culture seems less likely. With respect to hysteresis phenomena of biological systems there has been little work done in the literature (Grosz and Stephanopoulos, 1990). It is obvious that to understand the oscillation and hysteresis phenomena reported in this work more detailed analyses of the metabolic pathways and enzyme regulations are needed. In the next article of this series (Zeng et al., 1996), metabolic fluxes under oscillation conditions are analyzed and compared with results obtained from steady states under very similar environmental conditions. The reason(s) for the occurrence of oscillation and hysteresis can be localized.

## CONCLUSIONS

1. Sustained oscillation occurs in the continuous fermentation of glycerol by *K. pneumoniae,* which can be detected on-line by the periodic changes of $CO_2$ and $H_2$ production from exit gas analysis and by the change of alkali consumption rate. During oscillations, biomass concentration, specific growth rate, and dissolved fermentation products oscillate and/or fluctuate, some of them showing the same patterns and others showing shifted maxima and minima.
2. Sustained oscillation occurs only under conditions of substrate excess within a distinct region. At pH 7.0, it appears only to occur at a dilution rate above 0.15 $h^{-1}$ and below a substrate excess of about 110 g/L. At low, pH values, the culture becomes more likely to oscillate, even at a relatively low dilution rate and low substrate excess.
3. The operation mode was also found to be an important parameter in determining the stability and actual state of a culture, resulting in multiplicity and hysteresis under certain conditions, particularly at low pH values. Generally, a larger perturbation in substrate concentration, pH, and dilution rate may lead to oscillation and hysteresis.
4. Growth inhibition by products and/or substrate is not the prevailing mechanism for the oscillation, nor does oscillation originate from synchronous metabolism of the cell cycle. It appears that the oscillation and hysteresis observed in this work are due to genuine metabolic fluctuations of the microorganism.

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