Kinetic, Dynamic, and Pathway Studies of Glycerol Metabolism by *Klebsiella pneumoniae* in Anaerobic Continuous Culture: II. Analysis of Metabolic Rates and Pathways under Oscillation and Steady-State Conditions

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The oscillation phenomena reported in the preceding article for the anaerobic continuous fermentation of glycerol by *Klebsiella pneumoniae* are analyzed in terms of metabolic fluxes (metabolic rates and yields) and stoichiometry of pathways. Significant oscillations in the fluxes of CO_2, H_2, formic acid, ethanol, and reducing equivalents are observed which show obvious relationships to each other. Changes in the consumption or production rates of glycerol, acetic acid, 1,3-propanediol, and ATP are irregular and have relatively small amplitudes compared with their absolute values. By comparing the metabolic fluxes under oscillation and steady state that have nearly the same environmental conditions it could be shown that pyruvate metabolism is the main step affected under oscillation conditions. The specific formation rates of all the products originating from pyruvate metabolism (CO_2, H_2, formic acid, ethanol, acetic acid, lactic acid, and 2,3-butanediol) show significant differences under conditions of oscillation and steady state. In contrast, the specific rates of substrate uptake, ATP generation, and formation of products deriving either directly from glycerol (1,3-propanediol) or from the upstream of pyruvate metabolism (e.g., succinic acid) are not, or at least not significantly, affected during oscillation. Stoichiometric analysis of metabolic pathways confirms that other enzyme systems, in addition to pyruvate: formate–lyase, must be simultaneously involved in the pyruvate decarboxylation under both oscillation and steady-state conditions. The results strongly suggest oscillations of activities of these enzymes under oscillation conditions. It appears that the reason for the occurrence of oscillation and hysteresis lies in an unstable regulation of pyruvate metabolism of different enzymes triggered by substrate excess and drastic change(s) of environmental conditions.
Key words: oscillation • *Klebsiella pneumoniae* • glycerol metabolism • metabolic fluxes • pathway analysis

INTRODUCTION

In the preceding article (Menzel et al., 1996a), we reported on the oscillation and hysteresis phenomena found in the anaerobic continuous culture of *K. pneumoniae* grown on glycerol. Sustained oscillations could be observed for over 100 h in long-term cultivations, particularly at low pH values. The conditions for the occurrence and the patterns of cell growth and metabolism under oscillation have been characterized. It was found that sustained oscillation occurs only under conditions of substrate excess within a distinct regime. At pH 7.0, it seems to occur only at dilution rates higher than 0.15 h^{-1} and below a substrate excess of about 1100 mmol/L. At low pH values, the culture tends more likely to oscillate. In addition, the mode of changes of culture conditions was also found to be an important factor affecting the stability of the culture, resulting in multiplicity and hysteresis under certain conditions. Generally, a large perturbation of cultivation conditions, such as substrate concentration, dilution rate, and pH, may lead to sustained oscillation and hysteresis. These 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.

The patterns of growth and metabolism of *K. pneumoniae* under oscillation showed new features as compared with the oscillation phenomena reported in the literature (Clarke et al., 1988; Harrison and Topiwala, 1974; Jöbses et al., 1986; Porro et al., 1988). First, the anaerobic fermentation of glycerol is a fermentation process with multiple products. During oscillation the formation of some products, such as CO_2, H_2, formic acid, and ethanol, oscillate regularly and in certain relationships to each other and to the oscillation of cell growth; whereas other products such as acetic acid, 1,3-propanediol, and the substrate glycerol show relatively irregular oscillations and shifted maxima and minima (cf. Figs. 1 and 2 of Menzel et al. [1996a]). The magnitudes of the oscillations of the substrate and different products are also quite different. Oscillations of CO_2, H_2, formic acid,

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and ethanol are profound, whereas those of biomass, acetic acid, 1,3-propanediol, and glycerol are relatively small. Thus, cell cycle or synchronous growth could not be inferred as the main reason. Second, although the oscillation is induced by the change of environmental parameters, such as substrate concentration and pH, no definable parameters could be attributed to be responsible. As discussed by Menzel et al. (1996a) growth inhibition by substrate (glycerol) and inhibitory products is not the prevailing mechanism, at least not the intrinsic reason(s). Furthermore, a coexistence of two distinguished subpopulations in the culture, which is often the main reason for oscillations observed in mixed cultures and sometimes in pure culture (Clarke et al., 1988), seems to be less likely in the glycerol fermentation. It appears that possible intrinsic reason(s) should be sought in the intracellular metabolism, such as intracellular feedback regulation by intermediates and/or enzyme activities. To this end, measurements of intracellular metabolites and enzyme activities are necessary. In view of the large number of intracellular metabolites and enzymes involved in the glycerol fermentation it will be helpful to first localize the intracellular metabolites and regulation(s) causing these oscillation and hysteresis phenomena.

In this article, metabolic fluxes (rates and yields) under conditions of oscillation are analyzed and compared with results obtained from steady states under otherwise comparable environmental conditions, aiming at localizing the metabolic pathways and providing some evidence for the reason(s) for the occurrence of oscillation and hysteresis encountered in the anaerobic glycerol fermentation by *K. pneumonae*.

Metabolic Pathways of Anaerobic Glycerol Metabolism in *K. pneumoniae*

The metabolic pathway of glycerol fermentation has been mainly studied with *K. pneumoniae* (Forage and Foster, 1982; Lin, 1976; Neijssel et al., 1975; Streekstra et al., 1987). Figure 1 (solid lines) shows the possible metabolic pathways based on these results. Glycerol enters the cell by (facilitated) diffusion and can be converted into dihydroxyacetone, which is oxidized in the glycolysis pathway to pyruvate. Pyruvate reacts to the different fermentation products such as ethanol, acetic acid, 2,3-butanediol, and lactic acid known from fermentations of glucose. Part of the glycerol is dehydrated to 3-hydroxypropionaldehyde, which is reduced to 1,3-propanediol with regeneration of NAD (Lin, 1976). The physiological value of the 1,3-propanediol pathway is regeneration of $NADH_2$ formed during conversion of glycerol to dihydroxyacetone, formation of fermentation products, and biosynthesis. The enzymes catalyzing the conversion of glycerol to dihydroxyacetone or 3-hydroxypropionaldehyde and 1,3-propanediol have been studied by some researchers (Lin, 1976; Neijssel et al., 1975). However, there is no experimental work dealing with the enzymes which catalyze the formation of fermentation products deriving from dihydroxyacetone. These pathways

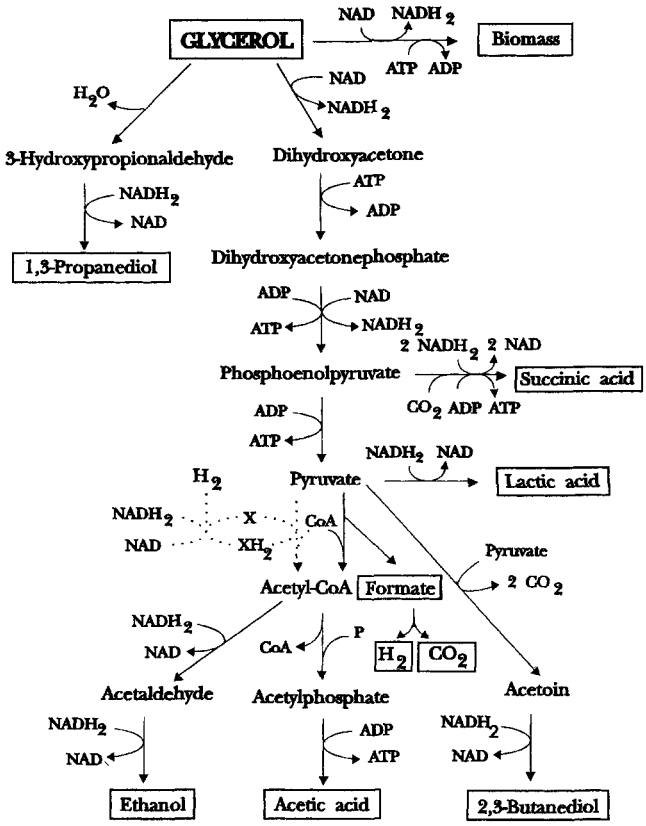

Figure 1. Pathways of anaerobic glycerol metabolism in *K. pneumoniae* according to Streekstra et al. (1987). Dotted lines proposed by Zeng et al. (1993).

have been taken from the well-known glucose metabolism without enzymatic examination. Zeng et al. (1993) showed that the reducing equivalents are not balanced according to the pathways suggested by Streekstra et al. (1987). They proposed the existence of further enzymes which participate in the conversion of pyruvate to acetyl-CoA such as pyruvate:ferredoxin oxidoreductase, NADH:ferredoxin oxidoreductase, and hydrogenase (dotted lines in Fig. 1).

MATERIALS AND METHODS

Organism

Klebsiella pneumoniae DSM 2026, obtained from the German Collection of Microorganisms (DSM, Braunschweig, Germany), was used in this study.

Cultivation Conditions and Analytical Methods

Culture medium compositions, cultivation conditions, and analytical methods were reported previously (Menzel, 1996a, 1996b).

Calculations

Calculation of the specific growth rate (μ):

$$\mu = \frac{1}{X} \cdot \frac{dX}{dt} + D$$

at steady state: $\frac{dX}{dt} = 0$ and $\mu = D$, where X is the biomass concentration.

Calculation of the specific product formation rate (q_P):

$$q_P = \frac{1}{X}\left(\frac{dC_P}{dt} + D \cdot C_P\right)$$

at steady state: $\frac{dC_P}{dt} = 0$ and $q_P = \frac{C_P \cdot D}{X}$, where C_P is the product concentration.

Calculation of the specific substrate uptake rate (q_s):

$$q_S = \frac{1}{X} \cdot \left(D \cdot C_S^{\text{med}} - D \cdot C_S - \frac{dC_S}{dt}\right)$$

at steady state: $\frac{dC_S}{dt} = 0$ and $q_S = \frac{D}{X} \cdot (C_S^{\text{med}} - C_S)$, where C_S and C_s^{med} are the substrate concentration in culture and medium.

Calculation of ATP and $NADH_2$ Formation Rate

According to the modified pathways shown in Figure 1, the reactions leading to the formation and consumption of ATP and $NADH_2$ can be written as follows (Zeng et al., 1993):

Glycerol → acetate + 2 ATP + (2 + x) $NADH_2$ + (1 − y) formate + y CO_2 + (y − x)H_2

Glycerol → ethanol + ATP + x $NADH_2$ + (1 − y) formate + y CO_2 + (y − x)H_2

Glycerol → ½ 2,3-butanediol + ATP + 1.5 $NADH_2$

Glycerol → lactate + ATP + $NADH_2$

Glycerol → succinic acid + ATP

Glycerol → 1,3-propanediol − $NADH_2$

where x is the fraction of reducing equivalents transferred to NAD during conversion from pyruvate to acetyl-CoA, and y is the fraction of formate split into CO_2 and H_2. For simplicity, ADP, NAD^+, and H^+ are omitted in the above reactions. The formation of biomass from glycerol is stoichiometrically written as:

$$4\ C_3H_8O_3 + 3\ NH_3 + \frac{3 \times 101}{Y_{ATP}}\ ATP \rightarrow 3\ C_4H_7O_2N + 8\ [H] + 6\ H_2O$$

where $C_4H_7O_2N$ denotes the elemental composition of biomass taken from Herbert et al. (1971). Y_{ATP} is the energetic yield of biomass (grams biomass/mole ATP). [H] represents the 1 mol of available hydrogen atom which is equivalent to 0.5 mol $NADH_2$. Thus, the above equation may be rewritten as:

$$4\ C_3H_8O_3 + 3\ NH_3 + \frac{3 \times 101}{Y_{ATP}}\ ATP \rightarrow 3\ C_4H_7O_2N + 4\ NADH_2 + 6\ H_2O$$

During steady state and oscillation the specific ATP formation rate q_{ATP} [mmol/(g · h)] is calculated as:

$$q_{ATP} = 2 \cdot q_{HAc} + q_{EtOH} + 2 \cdot q_{BD} + q_{Lac} + q_{Suc}$$

The specific formation rate of $NADH_2$ is calculated as:

$$q_{NADH_2}^{Prod} = 3 \cdot q_{HAc} + q_{EtOH} + 3 \cdot q_{BD} + q_{Lac} + 13.2 \cdot \mu - q_{H_2} - q_{For}$$

The specific consumption rate of $NADH_2$ is equal to the formation rate of 1,3-propanediol:

$$q_{NADH_2}^{Consum} = q_{PD}$$

Carbon and Reducing Equivalent Recoveries

The carbon recovery is calculated with the following equation:

$$C_{BK} = \frac{q_{HAc} + q_{EtOH} + 2q_{BD} + q_{Lac} + q_{PD} + q_{Suc} + 13.2 \cdot \mu}{q_{Glyc}}$$

The reducing equivalent recovery is calculated with the following equation:

$$R_H = \frac{q_{PD}}{3 \cdot q_{HAc} + q_{EtOH} + 3 \cdot q_{BD} + q_{Lac} + 13.2 \cdot \mu - q_{H_2} - q_{For}}.$$

RESULTS

Metabolic Rates and Yields Under Steady-State Conditions

Anaerobic continuous fermentation of glycerol by *K. pneumoniae* was carried out at pH 7.0 and different dilution rates (D = 0.1, 0.15, 0.25, 0.35, and 0.45 h^{-1}). At each dilution rate, steady states without oscillations were obtained at varying glycerol concentrations in the feed covering both glycerol-limiting and glycerol-excess conditions. Above a dilution rate of 0.15 h^{-1}, the glycerol concentration in the medium was increased carefully by relatively small steps to avoid oscillation of the culture (Menzel, 1996a). Figure 2 shows typical experimental results at a dilution rate of 0.35 h^{-1} as a function of glycerol concentration in medium (C_{Glyc}^{Medium}). Similar results were obtained at other dilution rates which are summarized in Tables I and II.

As can be seen in Figure 2 from the residual glycerol concentration in the reactor (C_{Glyc}) growth was essentially limited by substrate at C_{Glyc}^{Medium} up to ca. 820 mmol/L in this culture. This was further emphasized by the ratio of CO_2 and H_2 production (Fig. 2B), which had a value of nearly 1.0 under substrate limitation (Solomon et al., 1994). Under these conditions, the specific consumption rate of glycerol (q_{Glyc}) increased slightly with increasing glycerol concentration. In contrast, the specific formation rates of CO_2 (q_{CO_2}) and H_2 (q_{H_2}) declined. The specific formation rates of other fermentation products remained relatively constant. Increasing the glycerol concentration in medium beyond 820 mmol/L resulted in substrate excess. The residual substrate concentration increased rapidly with the feed concentration. Growth of cells was exclusively inhibited by the

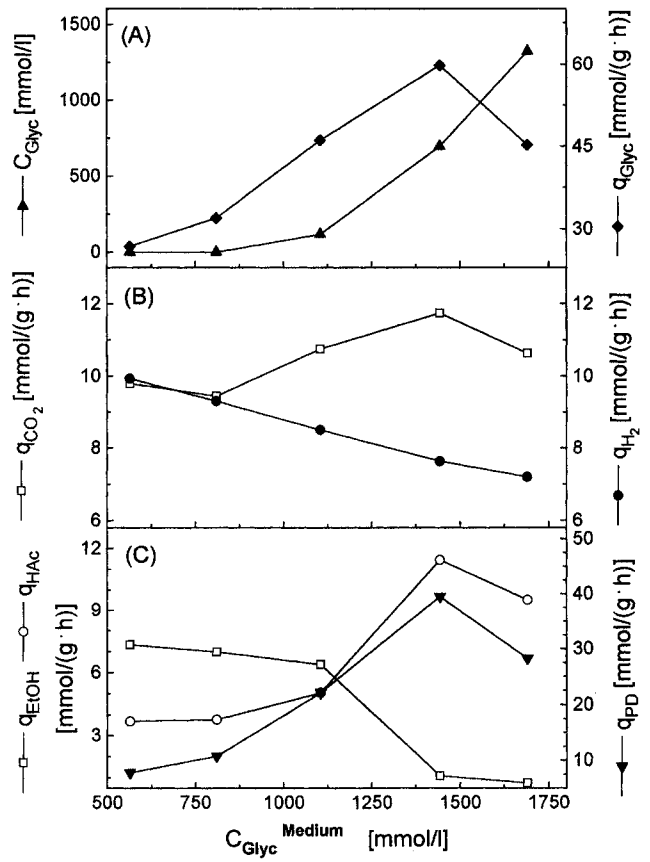

Figure 2. Experimental results of an anaerobic continuous culture of *K. pneumoniae* at a dilution rate of 0.35 h^{-1} and different glycerol concentrations in medium (pH 7). (A) Residual concentration (C_{Glyc}) and specific consumption rate (q_{Glyc}) of substrate; (B) specific formation rates of CO_2 (q_{CO_2}) and H_2 (q_{H_2}); and (C) specific formation rates of ethanol (q_{EtOH}), acetic acid (q_{HAc}), and 1,3-propanediol (q_{PD}).

accumulation of products and by the residual substrate under these conditions (Zeng et al., 1994). With the onset of substrate excess, q_{H_2} decreased further, whereas q_{CO_2} rose markedly with increasing substrate concentration. The specific consumption rate of glycerol (q_{Glyc}) and the formation rates of 1,3-propanediol (q_{PD}) and acetic acid (q_{HAc}) increased and leveled off at a relatively high concentration of residual glycerol. In contrast, the specific formation rate of ethanol (q_{EtOH}) declined. Under conditions of substrate excess, considerable amounts of lactic acid, succinic acid, 2,3-butanediol, and formic acid were also found in the culture. The specific formation rates of these minor products also increased with increasing C_{Glyc} (data not shown).

These results suggest that, for a comparison of metabolic rates, the nature of growth limitation and the substrate concentration need to be considered. Under conditions of relatively high substrate excess the metabolic rates are less significantly affected by substrate concentration. These are normally the conditions under which oscillations occur (Menzel et al., 1996a). Similar results are obtained for the product yields on glycerol (Tables I and II).

Metabolic Rates and Yields Under Conditions of Oscillation

The patterns of changes of substrate and product concentrations under oscillation have been reported and discussed in the preceding article (Menzel et al., 1996a). To gain more insight into these phenomena it is desirable to examine the experimental data in terms of specific rates of substrate consumption and product formation and the distribution (channeling) of substrate into different products. Typical changes of specific rates of substrate consumption and product formation are shown in Figures 3 and 4 for a culture at $D = 0.20\ h^{-1}$ and pH 7.0. In a similar way as done in Menzel et al. (1996a) the oscillation cycles are divided into two phases according to the evolution rates of CO_2 and H_2; i.e., one phase (phase I) with increasing gas evolution rates and another phase (phase II) with declining gas evolution rates. The specific formation rates of CO_2 (q_{CO_2}) and H_2 (q_{H_2}) oscillated in exactly the same patterns as the corresponding concentrations of CO_2 and H_2 in the off-gas (Menzel et al., 1996a), reaching their maxima and minima at the same points. As can be ascertained from Figures 3 and 4 the specific glycerol consumption rate and the formation rates of the major fermentation products all oscillated under these conditions. q_{Glyc} oscillated somewhat irregularly and had shifted maxima and minima compared to the oscillations of CO_2 and H_2 production rates. It can be shown that q_{Glyc} had also shifted maxima and minima compared to the concentration glycerol probably due to the oscillation of biomass concentration. The magnitude of q_{Glyc} oscillation was less profound compared to oscillations of q_{CO2}, q_{H2}, and μ, considering its relatively large absolute values. The specific formation rate of formic acid (q_{For}) displayed exactly the opposite patterns and was as significant as the gas evolution rates although its absolute values were relatively small. This is expected because CO_2 and H_2 are mainly released by the cleavage of formate. In contrast, the specific formation rate of ethanol changed in a similar way as the evolution rates of CO_2 and H_2, with the maxima being placed in advance of those of q_{CO_2}, q_{H_2}, and its concentration. The changes of the specific formation rate of acetic acid had similar patterns, but were not as regular as those for the gases and ethanol. The amplitude of q_{HAc} oscillation was also relatively low compared to its absolute values. The formation rate of the main fermentation product, 1,3-propanediol, showed very similar patterns as acetic acid. The maxima and minima of q_{HAc} and q_{PD} shifted considerably from those of their corresponding concentrations (cf. Fig. 2 of Menzel et al. [1996a]). The specific rates of the minor products, lactic acid, succinic acid, pyruvate, and 2,3-butanediol, were found to oscillate as well but had more irregular amplitudes and frequencies (data not shown).

Figure 5 shows the calculated specific ATP production rate during oscillations. The characteristics of q_{ATP} oscillation are somewhat similar to those of the substrate consumption and the acetic acid formation. No fixed oscillation pattern could be ascertained. Maximal values of q_{ATP} are

Table I. Experimental results of substrate-limited anaerobic continuous culture of *K. pneumoniae* at different dilution rates (pH 7).

D [h^{-1}]	C_{Glyc}^{Medium} [mmol/L]	q_{CO_2}	q_{EtOH}	q_{HAc}	q_{PD}	q_{Lac}	q_{BD}	q_{For}	q_{H_2}	C_{BK}	R_H	α^a	$Y_{CO_2/Glyc}$ [mol/mol]	$Y_{EtOH/Glyc}$ [mol/mol]	$Y_{HAc/Glyc}$ [mol/mol]	$Y_{PD/Glyc}$ [mol/mol]
0.15	564	9.8	7.35	3.70	7.79	0.06	0	0.27	9.93	1.00	0.99	0.92	0.43	0.32	0.14	0.34
0.20	514	10.89	7.46	4.70	13.43	0.12	0	0.41	11.43	0.96	1.03	0.97	0.39	0.27	0.17	0.49
0.25	295	13.57	9.31	7.21	17.40	0.07	0	0.72	14.53	1.01	0.95	0.92	0.39	0.27	0.21	0.50
0.35	223	15.91	10.47	10.06	22.80	0.05	0	1.68	16.04	1.00	1.06	0.86	0.31	0.21	0.20	0.45
0.35	443	18.72	12.46	10.03	27.43	0.53	0	2.23	18.59	1.01	1.04	0.93	0.32	0.21	0.17	0.47
0.45	183	15.95	9.10	14.25	25.06	0.09	0	2.29	16.57	1.04	0.76	0.81	0.33	0.19	0.30	0.52

Specific formation rates expressed in mmol/(g · h).

$^a\alpha = \dfrac{q_{H_2} + q_{For}}{q_{EtOH} + q_{HAc}}$.

generally found in phase I. However, a second maximum appears in phase II.

The distribution of substrate into different products during oscillation, globally expressed by the yield coefficients, is also calculated. Pronounced oscillations are obtained for the yields of CO_2, formic acid, and ethanol, all having exactly the same patterns as those of the specific rates. In contrast, the yields of acetic acid ($Y_{HAc/Glyc}$) and 1,3-propanediol ($Y_{PD/Glyc}$) show no obvious oscillation (data not shown). The changes of $Y_{HAc/Glyc}$ and $Y_{PD/Glyc}$ are more irregular than the corresponding specific rates.

Comparison of Metabolic Rates and Yields Under Steady State and Oscillation

As discussed by Menzel et al. (1996a) the oscillation patterns observed in the anaerobic glycerol fermentation by *K. pneumoniae* cannot be readily explained by mechanisms known so far. A comparison of metabolic fluxes under oscillation and steady-state conditions may give some clue for the peculiarities rising from the oscillation. Such a comparison is made possible by the fact that both steady states and sustained oscillations can be obtained under nearly the same environmental conditions depending on the operation mode. Figures 6 to 8 show comparisons of the specific formation rates of different products as functions of substrate concentration in medium for cultures carried out at a same dilution rate (0.35 h^{-1}). Similar results were obtained at a dilution rate of 0.25 h^{-1}. For the culture under oscillation, average values from an oscillation cycle were used. Although these values do not represent the instantaneous metabolic activities of cells under oscillation they may be considered as proper approximations of the average growth rate (= dilution rate)—especially for the specific rates of substrate consumption and of formation of products such as acetic acid and 1,3-propanediol, the oscillation amplitudes of which are relatively small as compared with their absolute values (cf. Figs. 3 and 4).

The results depicted in Figures 6 to 8 reveal that, although the specific rates of all products (CO_2, H_2, formic acid, ethanol, acetic acid, lactic acid, and 2,3-butanediol) originating from the pyruvate metabolism show significant differences under steady states and oscillation, the specific rates of 1,3-propanediol and succinic acid, which are either derived directly from glycerol or from the upstream of the pyruvate metabolism, have exactly the same values under the same substrate concentration. For the products originating from pyruvate metabolism generally much lower formation rates are obtained under oscillations than under steady states except for acetic acid. q_{HAc} under oscillation is nearly twice as high as that under steady state. This is probably due to the fact that the formation of acetic acid is

Table II. Experimental results of continuous culture of *K. pneumoniae* under conditions of substrate excess at different dilution rates (pH 7).

D [h^{-1}]	C_{Glyc}^{Medium} [mmol/L]	q_{CO_2}	q_{EtOH}	q_{HAc}	q_{PD}	q_{Lac}	q_{BD}	q_{For}	q_{H_2}	q_{Glyc}	C_{BK}	R_H	α^a	$Y_{CO_2/Glyc}$ [mol/mol]	$Y_{EtOH/Glyc}$ [mol/mol]	$Y_{HAc/Glyc}$ [mol/mol]	$Y_{PD/Glyc}$ [mol/mol]
0.10	1568	10.6	2.45	8.32	32.07	1.58	1.49	0.25	6.03	51.17	1.00	1.09	0.58	0.21	0.048	0.16	0.63
0.10	1795	11.21	2.60	6.80	28.18	3.70	1.31	0.27	6.41	46.62	1.02	1.05	0.71	0.24	0.055	0.15	0.60
0.15	1443	10.74	1.10	11.45	39.52	1.20	0.58	0.38	7.64	61.04	0.98	1.15	0.64	0.18	0.018	0.19	0.65
0.15	1690	10.63	0.76	9.52	28.23	1.37	0.66	0.58	7.20	45.77	0.99	0.96	0.76	0.23	0.017	0.21	0.62
0.10	804	10.39	3.87	7.20	24.83	1.06	0.80	0.30	8.01	39.71	1.00	1.05	0.75	0.26	0.097	0.18	0.63
0.25	909	21.36	6.67	10.11	57.26	1.57	4.70	0.49	8.44	88.73	0.99	1.04	0.53	0.24	0.075	0.11	0.65
0.35	870	22.94	10.14	10.40	67.57	3.94	6.01	6.02	11.58	112.5	0.99	1.21	0.86	0.09	0.090	0.09	0.60
0.45	607	15.43	1.69	20.17	64.19	0.86	0.61	5.04	10.17	97.26	0.98	1.15	0.70	0.16	0.017	0.21	0.66

Specific formation rates expressed in mmol/(g · h).

$^a\alpha = \dfrac{q_{H_2} + q_{For}}{q_{EtOH} + q_{HAc}}$.

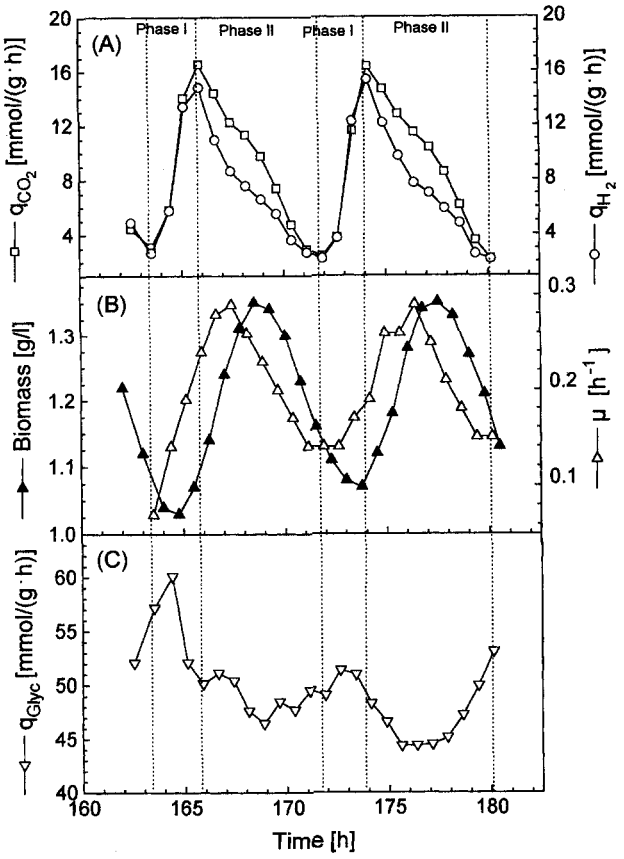

Figure 3. Experimental results of a continuous culture of *K. pneumoniae* under oscillation (D = 0.20 h^{-1} and pH 7). (A) Specific formation rates of CO_2 (q_{CO_2}) and H_2 (q_{H_2}); (B) biomass concentration and specific growth rate (μ); and (C) specific consumption rate of glycerol (q_{Glyc}).

associated with the production of an additional ATP and thus represents an advantage under oscillation (Fig. 1). These results indicate alternations of the pyruvate metabolism under oscillation. The pathways leading to the formation of 1,3-propanediol and succinic acid appear not to be affected. This coincides with the fact that the oscillations of q_{PD} and q_{Suc} are less significant compared to most of the other fermentation products (cf. Fig. 4).

The specific rates of glycerol uptake under steady state and oscillation conditions are also compared for cultures under nearly the same environmental conditions and are given in Table III for D = 0.35 h^{-1}. Two methods are used for the calculation of average q_{Glyc} under oscillation. Method I uses all the measured q_{Glyc} values from an oscillation cycle for the calculation. Method II calculates the average q_{Glyc} value as the average of the maximum and minimum of q_{Glyc} from an oscillation cycle. Slightly different values are obtained. From the results given in Table III it can be stated that the uptake rate of glycerol at a given dilution rate and glycerol concentration is not, at least not significantly, affected by the oscillation. This is also in accordance with the observation that the fluctuations of q_{Glyc} during oscillation are less significant compared to most of the fermentation products.

In Table III, the specific formation rates of ATP under steady state and oscillation are also compared. The two methods used above for the calculation of q_{Glyc} are also applied for q_{ATP} which give nearly the same values. It can be seen that q_{ATP} is not significantly affected by the oscillation. It should be mentioned that the calculated specific growth rate changes significantly during an oscillation cycle (Fig. 3). It is obvious that the large fluctuation of μ during oscillation is not coupled to accordant fluctuations in the substrate uptake and energy generation. This behavior may be attributed to the fact that cells under oscillation have an obvious excess of substrate (Menzel et al., 1996a). The rates of substrate uptake and ATP production are obviously under excess as well (Zeng and Deckwer, 1995). It is known, for a number of microorganisms, that consumption of substrate and energy for biosynthesis may only make up a small portion of the total consumption under substrate-sufficient conditions. This may also explain why q_{ATP} oscillates at a relatively low amplitude compared to μ (Fig. 5).

The distribution of glycerol into different products under oscillation also shows different patterns for different products compared to those under steady-state conditions. Whereas the yields of 1,3-propanediol and acetic acid have characteristics of glycerol metabolism under substrate excess, and slightly higher values during most of the period of oscillation, the yields of CO_2, ethanol, and formic acid are generally lower than those under both substrate limitation and substrate excess.

Stoichiometric Analysis of Metabolic Pathways

The aforementioned results reveal that only the pyruvate metabolism is significantly affected under oscillation. This is in accordance with the hypothesis that *K. pneumoniae* simultaneously uses more than one pyruvate decarboxylation enzyme system in the anaerobic glycerol fermentation (Zeng et al., 1993). These enzyme systems make the generation and consumption of reducing equivalents flexible. This in turn could lead to an accordant regulation of the formation of fermentation products. To characterize the involvement of different enzyme systems the parameter, α, which is slightly modified as follows, was found to be useful:

$$\alpha = \frac{q_{H_2} + q_{For}}{q_{EtOH} + q_{HAc}}$$

where α is a quantitative measure of the deviation of H_2 formation rate from that under the assumption that H_2 would only originate from formate cleavage (Fig. 1). If the enzymes hydrogenase and NAD(P)H:ferredoxin (flavodoxin) oxidoreductase are absent, and the TCA cycle is not involved, α represents the ratio of acetyl-CoA flux that is formed by pyruvate:formate-lyase to the total acetyl-CoA flux, having values between 0 and 1.0. Values of α lower than 1.0 imply an involvement of enzymes like pyruvate dehydrogenase or pyruvate:ferredoxin (flavodoxin) oxido-

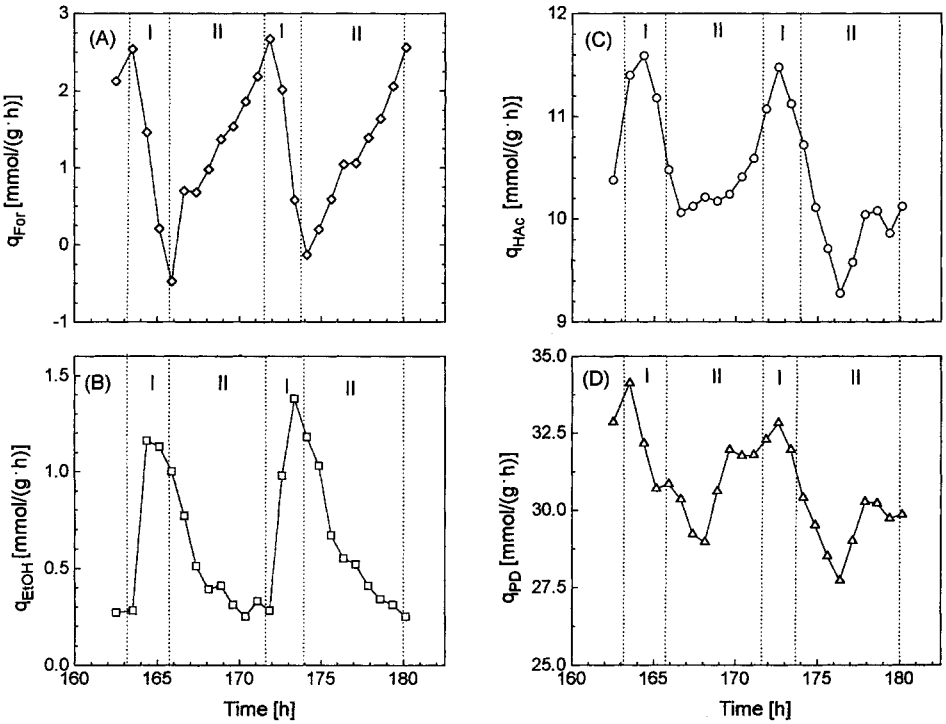

Figure 4. Experimental results of a continuous culture of *K. pneumoniae* under oscillation (D = 0.20 h^{-1} and pH 7). (A) Specific formation rate of formic acid (q_{For}); (B) specific formation rate of ethanol (q_{EtOH}); (C) specific formation rate of acetic acid (q_{HAc}); and (D) specific formation rate of 1,3-propanediol (q_{PD}).

reductase. Values of α higher than 1.0 indicate an involvement of hydrogenase or an enzyme like NAD(P)H:ferredoxin (flavodoxin) oxidoreductase. Values of α under steady states are compiled in Tables I and II. Values of α under steady states are affected by the nature of limitation. Generally, relatively high values of α (close to 1.0) are obtained for cultures under substrate limitation, indicating that pyruvate metabolism is mainly catalyzed by the enzyme system pyruvate:formate-lyase. Under conditions of substrate excess, values of α much smaller than 1.0 are obtained, indicating that pyruvate metabolism is catalyzed not only by the enzyme system pyruvate:formate-lyase. These conclusions are in general agreement with those reported previously (Zeng et al., 1993). Zeng et al. (1993) also found values of α obviously higher than 1.0 for culture under substrate limitation. It should be mentioned that the α value was defined slightly differently in Zeng et al. (1993) and that calculated values of q_{H_2} were used. q_{For} was also neglected. As pointed out recently by Zeng (1995), the calculation of q_{H_2} from a balance equation of reducing equivalents may be bound to relatively large error for the culture of *K. pneumoniae* under certain conditions.

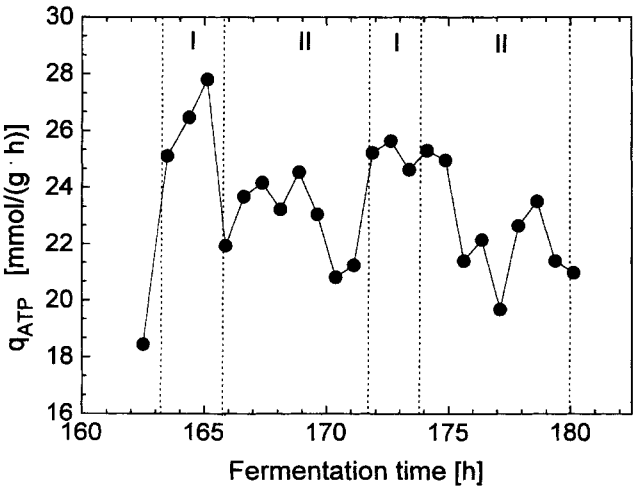

Figure 5. Specific formation rate of ATP (q_{ATP}) in a continuous culture of *K. pneumoniae* under oscillation (D = 0.20 h^{-1} and pH 7).

Figure 9 shows the change of α during oscillation. It clearly undergoes regular oscillation and follows exactly the patterns of q_{H_2}. These results strongly suggest oscillations of the enzyme systems involved in pyruvate metabolism, specifically in the decarboxylation of pyruvate to acetyl-CoA. The minimum values (0.43 to 0.45) reached are considerably lower than those of the steady states under substrate excess (Table II). A maximum as high as 1.28 is observed. It is not clear if this high α value is due to the involvement of hydrogenase and/or an enzyme like NAD(P)H:ferredoxin (flavodoxin) oxidoreductase, as previously suggested for cultures under substrate limitation (Zeng et al., 1993). It may also be possible that the total flux of acetyl-CoA under non-steady-state conditions is underestimated. The amount of acetyl-CoA consumed for lipid synthesis and possible TCA cycle activity is not considered in this work. Because the involvement of pyruvate decarboxylation enzymes such

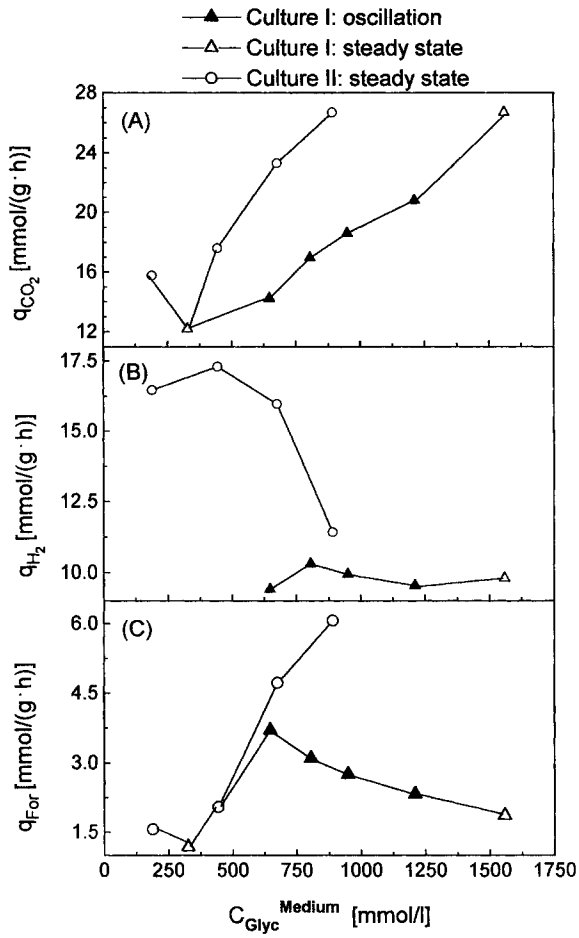

Figure 6. Comparison of metabolic rates under steady-state and oscillation conditions in continuous culture of *K. pneumoniae* at the same dilution rate (D = 0.35 h^{-1}) and different substrate concentrations in medium (pH 7). (A) Specific formation rate of CO_2 (q_{CO_2}); (B) specific formation rate of H_2 (q_{H_2}); and (C) specific formation rate of formic acid (q_{For}).

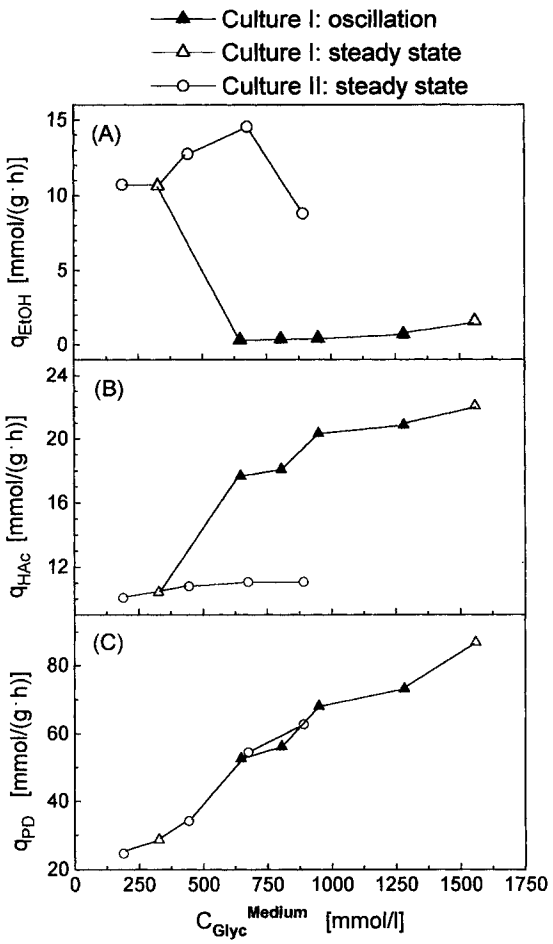

Figure 7. Comparison of metabolic rates under steady-state and oscillation conditions in continuous culture of *K. pneumoniae* at the same dilution rate (D = 0.35 h^{-1}) and different substrate concentrations in medium (pH 7). (A) Specific formation rate of ethanol (q_{EtOH}); (B) specific formation rate of acetic acid (q_{HAc}); and (C) specific formation rate of 1,3-propanediol (q_{PD}).

as pyruvate dehydrogenase and pyruvate:ferredoxin oxidoreductase is associated with generation of $NADH_2$ it is expected that the generation ($q_{NADH_2}^{Prod}$) and/or consumption ($q_{NADH_2}^{Consum}$) of reducing equivalents also oscillate. Indeed, distinct oscillations are found for $q_{NADH_2}^{Prod}$ and $q_{NADH_2}^{Consum}$ (data not shown). They oscillate in an opposite direction than that of α oscillation.

DISCUSSION

The present results demonstrate clearly that pyruvate metabolism involves the main reactions affected under oscillation conditions. In contrast, the specific rates of substrate uptake, ATP formation, and formation of products originating either directly from glycerol or from the upstream of pyruvate metabolism are insignificantly affected. The results also indicate that other enzyme systems, in addition to pyruvate:formate-lyase, must be simultaneously involved in pyruvate metabolism under both steady-state and oscillation conditions. The presence of pyruvate dehydrogenase in the pyruvate metabolism under anaerobic conditions has been recently enzymatically confirmed in our laboratory (unpublished data). Furthermore, the results presented suggest that the reason for the occurrence of oscillation seems to lie in the regulation of pyruvate metabolism and reducing equivalent balance.

Given the fact that more than one enzyme system is involved in pyruvate metabolism the above statement is not surprising. From the viewpoint of process dynamics (Gray and Scott, 1994; Ogunnaike and Ray, 1994), the occurrence of multiple steady states and/or instability of a nonlinear system requires the existence of at least two feedback control loops. The different pyruvate-decarboxylating enzyme systems with their feedback regulation mechanisms may represent such feedback control loops. If this is true then the phenomena of oscillation and hysteresis encountered in the *K. pneumoniae* culture might be explained as follows. Under normal conditions, such as under substrate limitation, the levels of involvement or activities of the pyruvate-decarboxylating enzyme systems may be relatively constant, resulting in steady states. Under these conditions, the

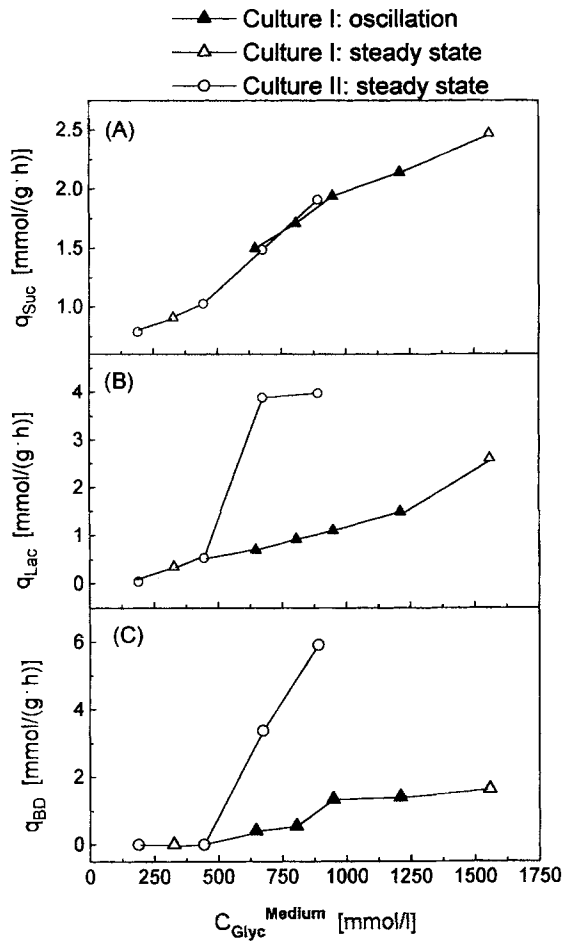

Figure 8. Comparison of metabolic rates under steady states and oscillation conditions in continuous culture of *K. pneumoniae* at the same dilution rate (D = 0.35 h^{-1}) and different substrate concentrations in medium (pH 7). (A) Specific formation rate of succinic acid (q_{Suc}); (B) specific formation rate of lactic acid (q_{Lac}); and (C) specific formation rate of 2,3-butanediol (q_{BD}).

activities of these enzyme systems (reflected by the α value) may depend mainly on the nature of growth limitation, residual substrate concentration, pH, and growth rate.

Upon smooth changes of culture conditions the relative activities of these enzymes may remain constant or be adjusted smoothly to catch up with the new conditions. However, if the conditions are changed drastically, such as by a large step change of substrate concentration and/or pH, the responses of two or more feedback regulation loops may lead to instability or oscillation due to time delays and overshoot regulations. It is known that some of the enzymes involved in the pyruvate metabolism and reducing equivalent regulation are sensitive to low pH values (Pecher et al., 1983; Tanisho et al., 1989). It is thus understood that the culture tends more likely to oscillate at low pH values (Menzel et al., 1996a). Furthermore, the relative activities or levels of enzymes governing the pyruvate decarboxylation and regulation of reducing equivalents under given environmental conditions may depend on the previous state of the culture, resulting in the hysteresis phenomena.

The previously discussed conjecture about the reason(s) for occurrence of oscillation in anaerobic glycerol fermentation by *K. pneumoniae* implies a mechanism by intracellular feedback regulation of enzyme activities. Different mechanisms such as induction, repression, and allosteric control of enzymes are possible. Whereas the environmental conditions such as concentrations of substrate and products may act as inducers or repressors some intermediates of the metabolism might also be involved. Harrison et al. (1969) observed sustained oscillation in intracellular $NADH_2$ in anaerobic continuous culture of *K. pneumoniae* (formerly *K. aerogenes*) grown on glucose.

It is imaginable that an oscillation of intracellular $NADH_2$ could cause oscillations in the enzymes governing the pyruvate decarboxylation and regulation of reducing equivalents. However, as pointed out by Harrison and Topiwala (1974), this kind of oscillation normally renders an oscillation frequency in the range of minutes rather than hours as observed in this work. For this reason, pure allosteric control of enzyme activity seems to be less likely the prevailing mechanism. It appears that the patterns of oscillation observed in this work are due to combinations of both feedback between cells and environmental parameters and intracellular regulations of two or more different enzyme systems catalyzing the same step of the reaction. The identification and quantitative assessment of activity and regulation of enzymes involved in the pyruvate metabolism and regulation of reducing equivalent balance are necessary steps in understanding ultimately the peculiarities of this

Table III. Comparison of substrate consumption rate and ATP formation rate under steady-state and oscillation conditions in continuous culture of *K. pneumoniae* at a constant dilution rate (D = 0.35 h^{-1}) and different substrate concentrations in medium.

Glycerol (medium) [mmol/L]	q_{Glyc} [mmol/(g · h)]			q_{ATP} [mmol/(g · h)]		
		Oscillation			Oscillation	
	Steady state	Method 1	Method 2	Steady state	Method 1	Method 2
729	92.32	82.72	86.67	40.73	38.32	38.36
803	91.39	81.59	85.76	44.85	38.71	43.4
870	105.89	96.48	97.83	42.13	40.28	40.11
948	100.48	87.25	90.32	48.39	41.47	43.81

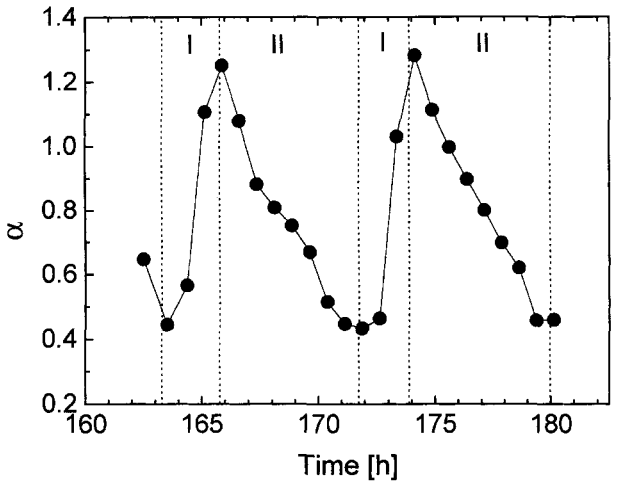

Figure 9. Oscillation of pyruvate metabolism as indicated by the ratio of acetyl-CoA flux catalyzed by pyruvate:formate-lyase to the total acetyl-CoA flux (α) in a continuous culture of *K. pneumoniae* under oscillation (D = 0.20 h^{-1} and pH 7). $\alpha = q_{H_2} + q_{For}/q_{EtOH} + q_{HAc}$.

bioprocess. To this end efforts are being made in our laboratory.

CONCLUSIONS

Pyruvate metabolism is the main step affected by oscillation during anaerobic metabolism of glycerol by *K. pneumoniae*. Under both steady-state and oscillation conditions enzyme systems in addition to pyruvate:formate-lyase must be simultaneously involved in pyruvate decarboxylation. Analyses of metabolic rates and stoichiometry of pathways strongly suggest oscillations of activities of these enzyme systems under oscillation conditions. The reason for the occurrence of oscillation and hysteresis in this culture appears to lie in the unstable regulation of pyruvate metabolism triggered by substrate excess and drastic pertubation(s) of environmental conditions.

NOMENCLATURE

C_{BK}	carbon recovery
C_{Glyc}^{Medium}	glycerol concentration in the feed medium
C_P	product concentration
C_S	substrate concentration
D	dilution rate
q_{ATP}	specific formation rate of ATP
q_{BD}	specific formation rate of 2,3-butanediol
q_{CO_2}	specific formation rate of CO_2
q_{EtOH}	specific formation rate of ethanol
q_{For}	specific formation rate of formic acid
q_{Glyc}	specific consumption rate of glycerol
q_{H_2}	specific formation rate of H_2
q_{HAc}	specific formation rate of acetic acid
q_{Lac}	specific formation rate of lactic acid
$q_{NADH_2}^{Consum}$	specific consumption rate of reducing equivalents ($NADH_2$)
$q_{NADH_2}^{Prod}$	specific production rate of reducing equivalents ($NADH_2$)
q_{PD}	specific formation rate of 1,3-propanediol
q_S	specific consumption rate of substrate
q_{Suc}	specific formation rate of succinic acid
R_H	recovery of reducing equivalents ($NADH_2$)
t	time
X	biomass concentration
Y_{ATP}	energetic yield of biomass
$Y_{CO_2/Glyc}$	mole yield of CO_2 based on glycerol consumed
$Y_{EtOH/Glyc}$	mole yield of ethanol based on glycerol consumed
$Y_{HAc/Glyc}$	mole yield of acetic acid based on glycerol consumed
$Y_{PD/Glyc}$	mole yield of 1,3-propanediol based on glycerol consumed
α	ratio of acetyl-CoA flux catalyzed by pyruvate:formate-lyase to the total acetyl-CoA flux
μ	specific growth rate

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