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# Microaerobic lysine fermentations and metabolic flux analysis

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**Abstract**

Oxygen supply is known to have an important influence on microaerobic production of amino acids, and several researches have shown that the behavior of many L-lysine-producing microorganisms under various aeration conditions are different. In order to investigate the fermentative behavior under microaerobic condition using *Corynebacterium glutamicum* ATCC 21253, several experiments were carried out where dissolved oxygen concentration was controlled at either 1% or 5%, as well as fully aerated condition. The calculation of intracellular metabolic fluxes was made to illustrate two kinds of metabolic characteristics observed in microaerobic cultures. Evaluated flux distributions indicated that the activities of TCA cycle enzymes decreased with the decrease in oxygen supply, resulting in the amplified phosphoenol pyruvate (PEP) carboxylation which contributed to the 30% of increase in lysine yield for the microaerobic culture at 5% DO concentration as compared with the case of aerobic fermentation. Further analysis indicates that NADPH may not be the yield-limiting factor, while low split-ratio of PEP carboxylation at PEP or aspartate branch at oxaloacetate is considered to limit lysine production under microaerobic conditions. 

*Keywords:* Lysine fermentation; Microaerobic culture; Cellular metabolism; Metabolic flux analysis

## 1. Introduction

Aeration conditions in bioreactors is an important operational variable for the production of desired bioproducts using microorganisms. Dissolved oxygen (DO) concentrations in the medium determined by the control policies of aeration have been shown to play essential role in the cell growth and have a variety of profound effects on cellular metabolism. For example, both growth rate and protein synthesis can be influenced by the level of DO [1,2]. Oxygen controls the microorganism by either stimulating or inhibiting the metabolic functions. Under aerobic conditions, oxygen serves primarily as a final hydrogen acceptor and is consumed by the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$) in the electron transport chain. Electron transport will be affected by the reduction in oxygen supply because of the reduced difference in the redox potential between the dehydrogenated couple and other electron acceptors. The hydrogen from the reduced carrier is usually transferred to other organic or inorganic compounds in the absence of oxygen, resulting in further reduction in ATP production [3]. The nutrient consumption and byproducts production rates are also sensitive to DO. The frequently observed trend is that the specific glucose consumption and lactate production rates increase, and oxygen consumption rate decreases when DO drops below certain critical value, known as microaerobic condition [2,4]. Despite these, microaerobic fermentation is favored by a number of important bioproducts including ethanol, 2,3-butanediol, xylitol, amino acids, antibiotics, vitamins, polysaccharides, enzymes and proteins [5]. The common problem for these fermentation is that anaerobic growth often leads to the formation of toxic fermentative by-products such as acetic acid and/or ethanol and thus reduction of biomass formation, while oversupply of oxygen may suppress the production of the desired product or enzyme activity.

Many researches on amino acid fermentation under different oxygen supply conditions have been carried out during the past two decades [6–9]. Fermentative performances can usually be improved using suitable control or optimization strategies when the effects of those environmental variables are well known. For example, in the work done by Radjai et al. [8], a 50% increase in the total amino acid yields could be achieved by automatic self-tuning control of redox potential at some low level using

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*Corynebacterium glutamicum* ATCC 14296. However, owing to the variety of microorganisms, fermentative behaviors under various aeration conditions have been found to be different for different strains. Akashi et al. [6] reported that the degree of the inhibition due to oxygen limitation was slight in the fermentative production of L-lysine using *Brevibacterium lactofermentum* 2256 and the production of L-threonine or L-isoleucine using *Brevibacterium flavum* 2247. In contrast, Hilliger and Hanel [7] demonstrated that the limitation of oxygen caused a decrease in L-lysine production using *Corynebacterium glutamicum* 9366. In the present work, we investigated the fermentation characteristics of another L-lysine producing microorganisms *Corynebacterium glutamicum* ATCC 21253 under different dissolved oxygen concentrations. Attention was paid especially to the microaerobic cultures with metabolic analysis since very few studies have been made so far on the microaerobic behavior of this strain.

On the other hand, intracellular metabolism of such microorganism as *Corynebacterium glutamicum* ATCC 21253 has been investigated in detail by several researchers [10–12], but all of those are at full oxygen supply condition. The methodology which makes use of stoichiometrically based mass balances to determine the intracellular flux distributions is often employed in those metabolic analysis. This metabolic flux analysis technique has been readily applied to many other processes since less detailed information regarding enzyme kinetics is required [10,13–16]. The basis of metabolic flux analysis is the stoichiometry of all relevant biochemical reactions of the system under study. From steady-state balancing of input–output and intermediate compounds, a set of linear balance equations is obtained [17]. Since the solution to balance equations gives the quantitative relationship between conversion rates of extracellular metabolites and intracellular enzymatic reaction rates, significant information regarding potential metabolic bottlenecks may be obtained from metabolic flux analysis. Consequently, the analysis may indicate the precise locations in the pathway where genetic scientists should modify to overproduce some key enzymes or repress unfavorable paths and then increase the amount of the desired products. In the present research, therefore, we focused on microaerobic lysine fermentation using *Corynebacterium glutamicum* ATCC 21253, and metabolic flux analysis was made to examine the intracellular flux distributions and quantitatively understand the metabolism under microaerobic condition.

## 2. Materials and methods

### 2.1. Organism and cultivation

The strain used was *Corynebacterium glutamicum* ATCC 21253. This organism is auxotrophic for both L-homoserine (or L-threonine plus L-methionine) and L-leucine, and produces lysine under threonine limitation. The seed culture was conducted using a 50 ml L-shaped test tube with 15 ml of LB5G medium (5 g $l^{-1}$ glucose, 5 g $l^{-1}$ yeast extract, 10 g $l^{-1}$ tryptone and 5 g $l^{-1}$ NaCl). The test-tube cultivation was carried out in the 30°C water bath for 18 h, and the culture broth was then transferred into a 500 ml T-shaped flask containing 150 ml of medium, where further cultivation was made at 30°C with 150 rpm for preculture. After 24 h, the broth was transferred into a 5 l jar-fermentor (MDL500, Marubishi, Tokyo) with a working volume of 3 l, and the cultivation was carried out for about 70 h. The compositions of the media were slightly different from those given in Vallino and Stephanopoulos [10]. The preculture medium was prepared in the present study in two parts (for 1 l medium): (A) 20 g glucose, 1.14 g citrate·$Na_3$·$2H_2O$, 55 mg $CaCl_2$, 200 mg $MgSO_4$·$7H_2O$, 20 mg $FeSO_4$·$7H_2O$, 1 g NaCl and 100 ml concentrated salts solution, adjusted to pH 5.0 with HCl; (B) 8 g $K_2HPO_4$, 1 g $KH_2PO_4$, 150 mg threonine, 100 mg leucine, 40 mg methionine, 0.5 mg biotin, 1 mg thiamine·HCl and 5 g $(NH_4)_2SO_4$. The fermentative medium was composed of (in 1 l medium): (A) 150 g glucose, 1 g citric acid, 0.6 g $MgSO_4$·$7H_2O$, 50 mg $FeSO_4$·$7H_2O$, 2 g NaCl, 1 g $CaCl_2$ and 20 ml concentrated salts solution, adjusted to pH 4.0 with HCl; (B) 4 g $K_2HPO_4$, 2 g $KH_2PO_4$, 733 mg threonine, 1.5 g leucine, 600 mg methionine, 1 mg biotin, 2 mg thiamine·HCl and 40 g $(NH_4)_2SO_4$. The compositions of concentrated salts solution were: 200 mg $l^{-1}$ $FeCl_3$·$6H_2O$, 200 mg $l^{-1}$ $MnSO_4$, 50 mg $l^{-1}$ $ZnSO_4$·$7H_2O$, 20 mg $l^{-1}$ $CuCl_2$·$2H_2O$, 20 mg $l^{-1}$ $Na_2B_4O_7$·$10H_2O$ and 10 mg $l^{-1}$ $(NH_4)_6Mo_7O_{24}$·$4H_2O$, adjusted to pH 1.5 with HCl. The pH value of the process was monitored using a pH sensor (FC-1, Tokyo Rikakikai, Tokyo) and maintained at 7.0 by addition of 25% w/w ammonium hydroxide. The temperature was controlled at 30°C. The dissolved oxygen concentration was monitored with an online DO sensor (DO-1, Tokyo Rikakikai, Tokyo) and controlled at the desired value by adjusting the agitation rate and the flow rate of filter-sterilized air. A laser turbidimeter (LA 300LT, ASR, Tokyo) and a $CO_2/O_2$ gas analyzer (LX-750, Iijima Electronics MFC, Tokyo) were used to monitor the biomass concentration and the carbon dioxide/oxygen concentrations in the exhaust gas, respectively.

### 2.2. Assays

Off-line measurement of cell concentration was carried out using a spectrophotometer (Ubest-30, Jasco, Tokyo) at 660 nm with the appropriate dilution. The value of optical density was converted to dry cell weight (DCW) using relationship where 1 $OD_{660}$=0.28 g DCW $l^{-1}$. Glucose concentration was measured with an enzyme kit (Wako Pure Chemicals, Osaka). Concentrations of amino acids were measured by a Waters HPLC equipped with a BioRad (Richmond, CA) Model 450 column, organic acids were detected by HPLC (LC10A, Shimadzu, Kyoto) equipped with a Shim-pack SCR-102H column.

### 2.3. Estimation of metabolic fluxes

The metabolic networks can be written to facilitate the construction and analysis of intracellular metabolic bioreactions and to estimate flux distribution in such networks from measured experimental data. Since the flux value indicates the activity of the corresponding enzymatic reaction, valuable intracellular information may be obtained to characterize the culture under different environmental conditions. The ideas concerning the construction of metabolic flux model, the solution method for obtaining flux distributions with consistency analysis may be found elsewhere [14,18]. As to the biochemistry of *Corynebacterium glutamicum* ATCC 21253, several researchers [10,19] have studied carefully about this strain in aerobic fermentation. The stoichiometric equations are given in Table 1 with some modification from those given in Vallino and Stephanopoulos [10]. Those equations have been identified by a variety of biochemical analyses carried out for *C. glutamicum* and have been employed to provide a structured biochemical model for the flux analysis. The typical metabolic pathways of *C. glutamicum* ATCC 21253 are schematically illustrated in Fig. 1. The fluxes of the metabolic network can be calculated in accordance with the mass/energy balances around each relevant metabolite as given in Table 2. The matrix representation for stoichiometric equations can be expressed as follows:

$$\mathbf{Ar} = \mathbf{q} \tag{1}$$

where $\mathbf{A}$ is the stoichiometric coefficient matrix ($36\times34$) which comprises the coefficients of all metabolites in all enzymatic reactions. $\mathbf{r}$ is a ($34\times1$) metabolic flux vector and $\mathbf{q}$ is the specific conversion rate vector with the dimension of 36. The weighted least squares solution for $\mathbf{r}$ can be obtained in the form of

$$\mathbf{r} = (\mathbf{A}^{\mathrm{T}}\psi^{-1}\mathbf{A})^{-1}\mathbf{A}^{\mathrm{T}}\psi^{-1}\mathbf{q} \tag{2}$$

where $\psi$ ($36\times36$) is the diagonal variance-covariance matrix of measurement noise associated with $\mathbf{q}$ vector. The diagonal elements of $\psi$ associated with extracellular metabolites were determined by the standard deviations of the measured values, i.e. 10% for biomass, CER and OUR, and 5% for other measurements. Other diagonal elements corresponding to intracellular metabolites were set to 0 or some extremely small value. It is known that the metabolic flux calculation can be well performed for steady-state process. Although for non-steady state process the possible accumulation of the metabolites in the cell body brings the inconvenience to flux estimation using above method, the pseudo-steady state (PSS) approximation is usually used for intracellular metabolites [10,13,14]. The reason for using PSS assumption was well elucidated by Vallino and Stephanopoulos for the similar process [10], which pointed out that for the most intracellular metabolites, since the intracellular volume is much smaller than the extracellular volume, the difference between flows producing and

Table 1
Biochemical reactions in *C. glutamicum* (modified from Vallino and Stephanopoulos [10])

Glucose transport
(1) GLU+PEP=G6P+PYR

EMP pathway
(2) G6P=FRU6P
(3) FRU6P+ATP=2 GAP+ADP
(4) GAP+ADP+NAD=NADH+G3P+ATP
(5) G3P=PEP+H2O
(6) PEP+ADP=ATP+PYR

Pentose phosphate pathway
(7) G6P+H2O+2 NADP=RIBU5P+CO2+2 NADPH
(8) RIBU5P=R5P
(9) RIBU5P=XYL5P
(10) XYL5P+R5P=SED7P+GAP
(11) SED7P+GAP=FRU6P+E4P
(12) XYL5P+E4P=FRU6P+GAP

TCA cycle
(13) PYR+COA+NAD=ACCOA+CO2+NADH
(14) ACCOA+OAA+H2O=ISOCIT+COA
(15) ISOCIT+NADP=AKG+NADPH+CO2
(16) AKG+COA+NAD=SUCCOA+CO2+NADH
(17) SUCCOA+ADP=SUC+COA+ATP
(18) SUC+H2O+FAD=MAL+FADH
(19) MAL+NAD=OAA+NADH

Glutamate, glutamine production
(20) NH3+AKG+NADPH=GLUT+H2O+NADP
(21) GLUT+NH3+ATP=GLUM+ADP

Byproducts formation or utilization[a]
(22i) PYR+NADH=LAC+NAD
(22ii) LAC+FAD=PYR+FADH
(23) ACCOA+ADP=AC+COA+ATP
(24) AKG=AKGE
(25) PYR+GLUT=ALA+AKG
(26) 2 PYR+NADPH+GLUT=VAL+CO2+H2O+NADP+AKG

Oxidative phosphorylation[b]
(27) 2 NADH+O2+2 (P/O) ADP=2H2O+2 (P/O) ATP+2 NAD
(28) 2 FADH+O2+(P/O) ADP=2H2O+(P/O) ATP+2 FAD

Anaplerotic reaction
(29) PEP+CO2=OAA

ATP consumption for maintenance and futile cycles
(30) ATP=ADP

Lysine synthesis
(31) OAA+GLUT=ASP+AKG
(32) ASP+PYR+2NADPH+SUCCOA+GLUT+ATP=
SUC+AKG+CO2+COA+LYSI+2 NADP+ADP
(33) LYSI=LYSE

Biomass formation
(34) 0.021 G6P+0.007 FRU6P+0.09 R5P+0.036 E4P+0.013
GAP+0.15 G3P+0.052 PEP+0.03 PYR+0.332 ACCOA+0.08
ASP+0.033 LYSI+0.446 GLUT+0.025 GLUM+0.054 ALA+0.04
VAL+3.82 ATP+0.476 NADPH+0.312 NAD=BIOMAS+3.82
ADP+0.364 AKG+0.476 NADP+0.312 NADH+0.143 CO2

[a]Only one reaction between the formation and utilization of lactic acid was used for a specified phase.
[b]Various values of P/O ratio were used for different fermentation, i.e. 0.6, 1.36 and 2.0 [3] for microaerobic cultures with 1%, 5% DO concentrations and aerobic fermentation, respectively.

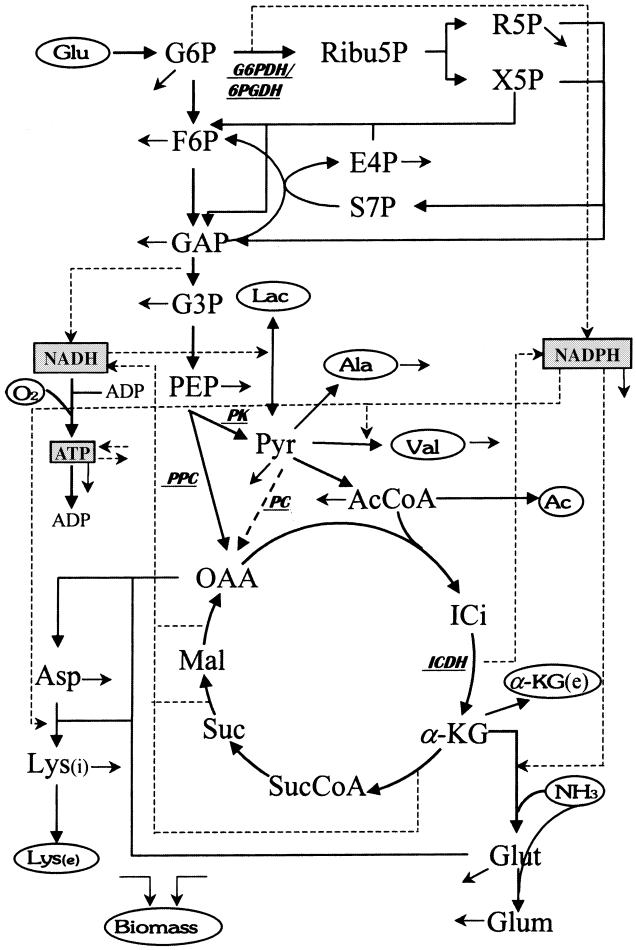


Fig. 1. Biochemistry of *C. glutamicum* ATCC 21253 during lysine production. Abbreviations of enzymes: G6PDH: glucose 6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase; PK: pyruvate kinase; PPC: phosphoenol pyruvate carboxylase; PC: pyruvate carboxylase; ICDH: isocitrate dehydrogenase. Abbreviations of metabolites are shown in Table 2.

consuming the metabolite would be less than 10% even if an intracellular metabolite accumulation rate of 1 mM $min^{-1}$ occurred, which would significantly alter enzyme kinetics in less than 1 min. Therefore the PSS approximation (i.e. zero conversion rate) was applied to 26 intracellular metabolites, namely, all the metabolites given in Table 2 except AC, AKGE, ALA, BIOMAS, CO2, GLU, LAC, LYSE, O2 and VAL. The important fluxes in vector **r** can then be identified and utilized for subsequent metabolic analysis.

## 3. Results and discussion

### 3.1. Fermentation

Several experiments were carried out using a 5 l jar-fermentor with several different DO concentrations, namely, aerobic and microaerobic fermentation. For microaerobic cultures, two different cases, namely 1% (MA1) and 5% (MA2) dissolved oxygen concentrations were considered. Typical experimental results of aerobic fermentation (AF) and two kinds of microaerobic fermentation are shown in Figs. 2 and 4, respectively. For better understanding of the batch lysine fermentation, the fermentation period may be divided into several phases corresponding to the different physiological states. The fermentation phases portrayed in Figs. 2–4 can be briefly described in the followings. Phase I is mainly marked by cell growth. For aerobic fermentation, little or no byproducts were produced in this phase, while lactic acid was secreted markedly in this growth phase under microaerobic conditions. The duration of this phase is considered to be governed by the consumption of threonine from the initial supply. When the threonine concentration in the medium is decreased to some low level, phase II starts with the high lysine secretion rate since the inhibition of aspartate kinase by threonine and lysine is alleviated. Taking into account the diversification of the fermentation processes, this phase is regarded to end before the decay of cells. To make the calculation more precise, two different stages, namely the early and late stages were considered corresponding to the late exponential growth and stationary growth parts in phase II. The duration of this phase is

Table 2
Abbreviations of metabolites

| | | |
|---|---|---|
| AC: Acetate; | ACCOA: Acetyl coenzyme A | AKG: $\alpha$-ketoglutarate |
| AKGE: $\alpha$-ketoglutarate (extracellular) | | ALA: Alanine; |
| | | ASP: Aspartate |
| ATP: Adenosine 5′-triphosphate | | BIOMAS: Biomass |
| CO2: Carbon dioxide | | E4P: Erythrose-4-phosphate |
| FADH: Flavin adenine dinucleotide, reduced | | FRU6P: Fructose-6-P |
| G3P: 3-phosphoglycerate | | GAP: Glyceraldehyde-3-P |
| GLU: Glucose; | G6P: Glucose-6-P | GLUM: Glutamine |
| GLUT: Glutamate | | ISOCIT: Isocitrate |
| LAC: Lactate; | LYSE: Lysine (extracellular) | LYSI: Lysine (intracellular) |
| MAL: Malate; | NADH: Nicotinamide adenine dinucleotide, reduced | NADPH: Nicotinamide adenine dinucleotide phosphate, reduced |
| O2: Oxygen; | OAA: Oxaloacetate | PEP: Phosphoenol pyruvate |
| PYR: Pyruvate; | R5P: Ribose-5-P | RIBU5P: Ribulose-5-P |
| SED7P: Sedoheptulose-7-P | | SUC: Succinate |
| SUCCOA: Succinate coenzyme A; | VAL: Valine | XYL5P: Xylulose-5-P |

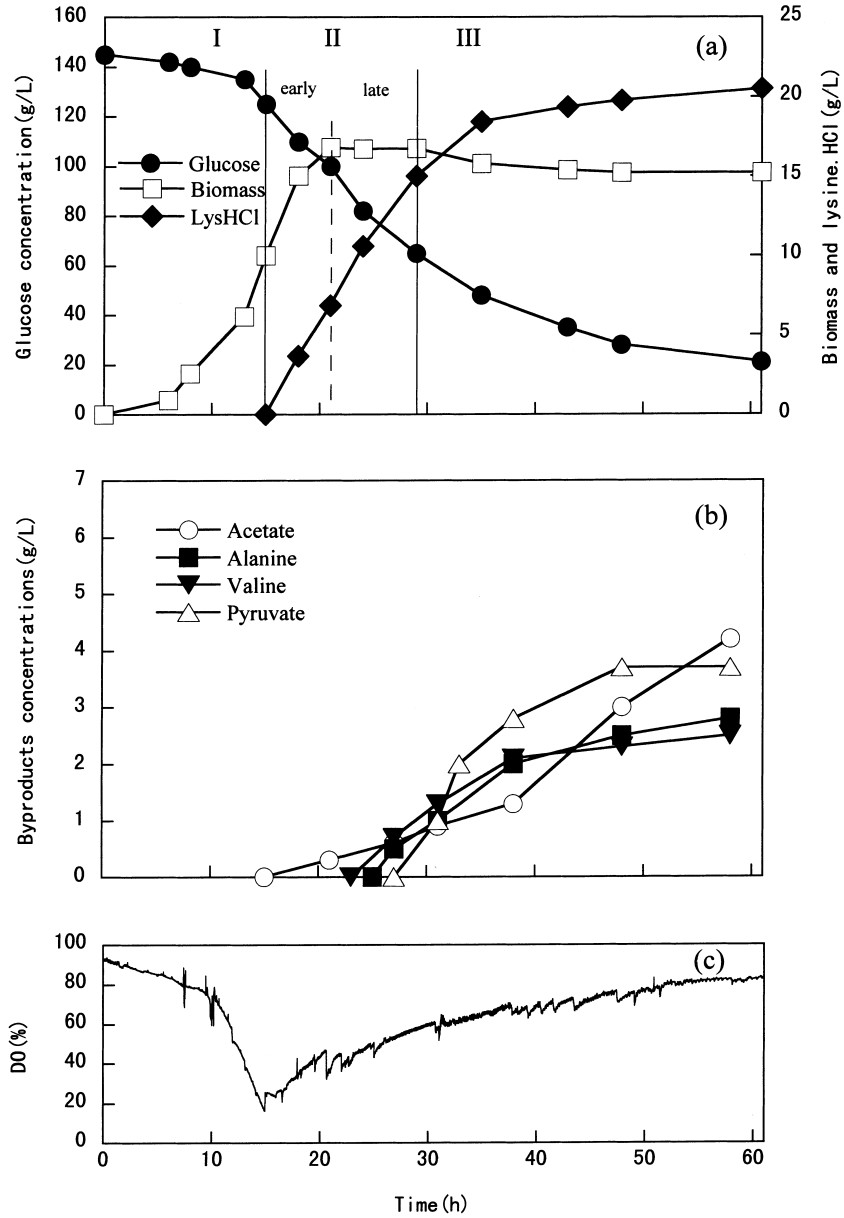


Fig. 2. Time courses of aerobic fermentation using *C. glutamicum* ATCC 21253. (a) profiles of glucose (●), biomass (□) and lysine·HCl (◆). (b) Profiles of acetate (○), alanine (■), valine (▼) and pyruvate (△). (c) Profile of DO concentration.

thought to be dependent on the experimental conditions to a greater extent. Phase III represents the death phase with the decrease in biomass concentration and accumulation of some byproducts.

Some results extracted from Figs. 2–4 are summarized in Table 3. It could be found that the maximum cell concentrations in microaerobic cultures were 12–13 g $l^{-1}$, which corresponds to about 80% of the concentration in aerobic fermentation, so that the aerobic cultivation gave the highest specific growth rate. Glucose consumption rates were also low under oxygen limited conditions where the lactic acid significantly accumulated in microaerobic fermentation in particular for the final lactic acid concentration of about 39 g $l^{-1}$ in the case where DO concentration was 1%. In case of 5% DO concentration, the lactic acid concentration in the broth decreased from the maximum of about 10 g $l^{-1}$ at 40 h to the final value of 2.3 g $l^{-1}$. However, α-ketoglutarate, one of the TCA metabolites, secreted markedly in this case with the final concentration of 16 g $l^{-1}$, more than two-fold of that in the culture with 1% DO concentration. In almost all cases, lysine yields in phase II, especially the early stage of phase II, were higher than the other phases, though the yields were different for various DO conditions. Although little or no lysine secretion could be seen in phase III of aerobic fermentation, some lysine production could be seen in this phase of microaerobic cultures. This phenomenon was in particular obvious for the case where DO concentration was controlled at 5% and lysine molar yield

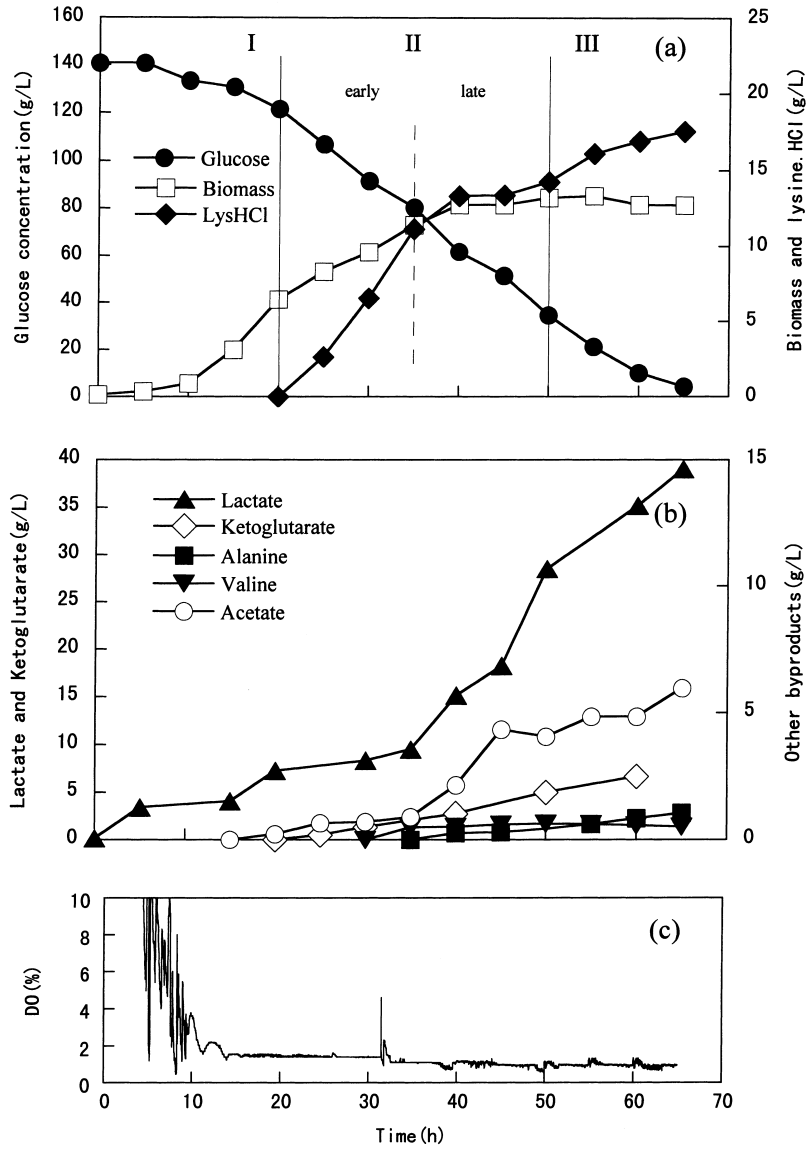


Fig. 3. Time courses of microaerobic fermentation with 1% DO concentration using *C. glutamicum* ATCC 21253. (a) profiles of glucose (●), biomass (□) and lysine·HCl (◆). (b) Profiles of lactate (▲), alanine (■), valine (▼), acetate (○) and α-ketoglutarate (◇). (c) Profile of DO concentration.

Table 3
Comparison of the phases in cultures with different DO concentrations. MA1: microaerobic culture with 1% DO; MA2: microaerobic culture with 5% DO; AF: aerobic fermentation. Concentration: g $l^{-1}$; Lysine yield: mol $mol^{-1}$ glucose $h^{-1}$; Specific growth rate: $h^{-1}$ and Duration: h

| Parameters | MA1 | MA2 | AF |
|---|---|---|---|
| Maximum cell concentration | 13 | 12 | 16 |
| Specific growth rate in phase I | 0.15 | 0.24 | 0.35 |
| Final lactate concentration | 39 | 2.3 | 0 |
| Final α-ketoglutarate concentration | 7 | 16 | 0 |
| Lysine yield on glucosephase | | | |
| Phase I | 0 | 0 | 0 |
| Phase II (early) | 0.24 | 0.38 | 0.3 |
| Phase II (late) | 0.14 | 0.18 | 0.22 |
| Phase III | 0.10 | 0.31 | 0.02 |
| Overall lysine yield | 0.13 | 0.17 | 0.13 |
| Duration of phase II | 30 | 15 | 13∼15 |

was observed to be around 30% in phase III. Overall lysine yield on glucose in the case of 5% DO concentration was about 30% and is higher than those for the other two cases. The duration of phase II in the typical aerobic culture is less than 15 h, while this duration tends to increase when DO concentration in the medium decreases.

### 3.2. Interpretation of metabolic characteristics using metabolic network model

Intracellular enzymatic reaction rates at specified time interval may be used to analyze the metabolism under different culture conditions. Before the calculation of specific conversion rates for extracellular metabolites, the first order digital filter was designed to smooth the on-line data (oxygen and carbon dioxide concentrations in off-gas) and

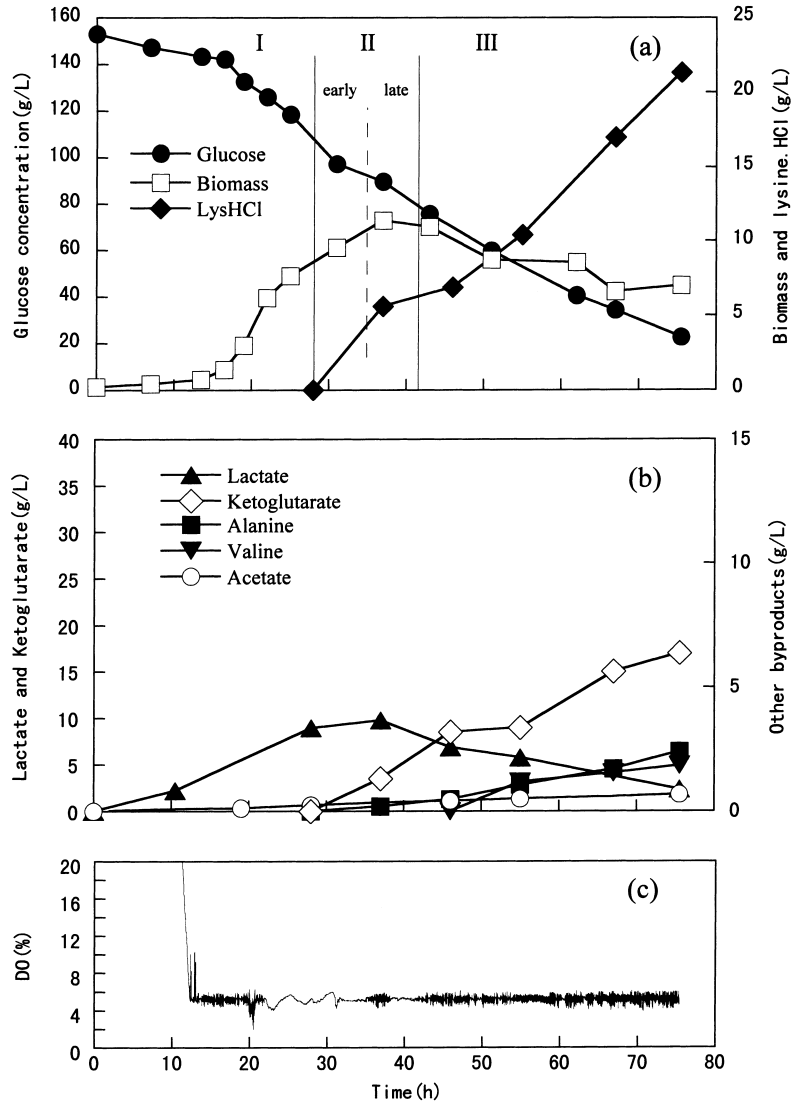


Fig. 4. Time courses of microaerobic fermentation with 5% DO concentration using *C. glutamicum* ATCC 21253. (a) profiles of glucose (●), biomass (□) and lysine·HCl (◆). (b) Profiles of lactate (▲), alanine (■), valine (▼), acetate (○) and $\alpha$-ketoglutarate (◇). (c) Profile of DO concentration.

off-line data (concentrations of biomass, glucose, lysine and byproducts). Then, the specific conversion rates obtained from the smoothed data were utilized for the flux calculation for each enzymatic reaction with the metabolic network model.

### 3.2.1. Functions of anaplerotic route

During lysine fermentation using *C. glutamicum*, most carbon is expected to be diverted towards the synthesis of intracellular oxaloacetate (OAA), the important precursor for lysine synthesis. The most possible pathway for the generation of OAA should be the reaction via phosphoenol pyruvate carboxylase (PPC), which converts phosphoenol pyruvate (PEP) with $CO_2$ to OAA (reaction 29 in Table 1). The fact of the absence of isocitrate lyase activity implies that glyoxylate bypass will not function as an anaplerotic route when glucose is used as the main carbon source [10]. Although the degree of induction of pyruvate carboxylation (converting pyruvate with $CO_2$ and ATP to OAA as shown in Fig. 1) still remains ambiguous [12,19], the effect of anaplerotic route through pyruvate carboxylase (PC) is actually identical with that through PPC except some differences of local flux distributions relating the intracellular PEP and pyruvate [12]. Therefore, in the present flux calculation, only the pathway through PPC was considered as an anaplerotic route for replenishing the TCA cycle with OAA, which is constantly removed from the cycle for production of aspartate family of amino acids.

Fig. 5 shows how the fluxes of anaplerotic route change with culture phases. Namely, in phase I, the anaplerotic fluxes were almost the same as those in early phase II, and the carbon source transferred from PEP through PPC may be considered to be exclusively utilized for biomass synthesis, since in such phases no lysine was produced due to the inhibition of aspartate kinase. It may be said that the anaplerotic fluxes and the specific growth rates exhibited

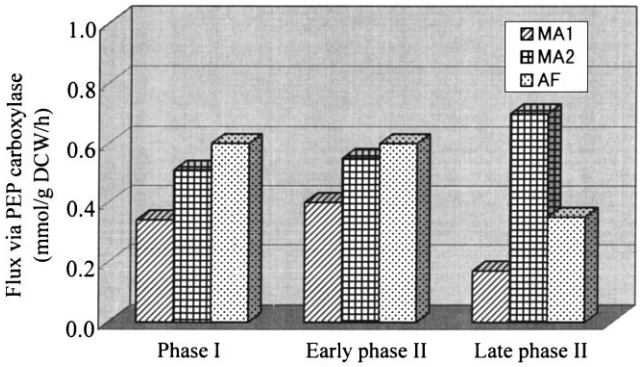


Fig. 5. Comparison of fluxes via PEP carboxylase. MA1: microaerobic culture with 1% DO concentration; MA2: microaerobic culture with 5% DO concentration; AF: aerobic fermentation.

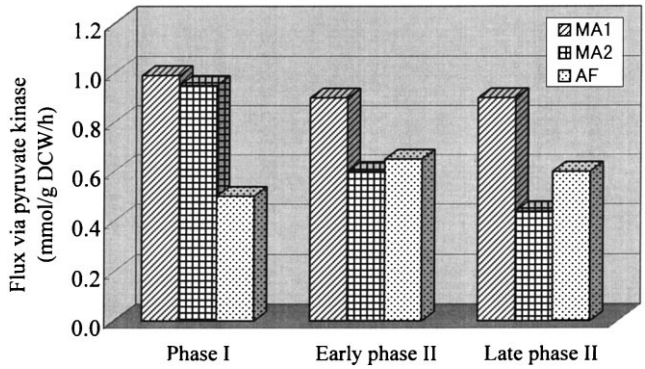


Fig. 6. Comparison of fluxes via pyruvate kinase. MA1: microaerobic culture with 1% DO concentration; MA2: microaerobic culture with 5% DO concentration; AF: aerobic fermentation.

similar changing trends in phase I for each DO concentration in the medium. In other words, the anaplerotic flux may be closely related to the cell growth in phase I. On the contrary, in both stages of phase II, the anaplerotic route functioned mainly for the formation of aspartate from OAA, resulting in the secretion of lysine. Only small amount of OAA formed from PEP carboxylation was used for biosynthesis. At the same time, in case of the secretion of the TCA cycle metabolites, it would be more important to replenish OAA from anaplerotic route to maintain the TCA cycle. Consequently, the flux via PPC for the case of microaerobic culture with 5% DO concentration was high as compared with the other two cases (see Fig. 5) due to the secretion of α-ketoglutarate as will be illustrated later. In such phases, anaplerotic route could not be used to reflect a certain physiological process such as biosynthesis or lysine production. On the other hand, it could be found from Fig. 5 that the anaplerotic fluxes for the case where DO concentration was 1% were obviously lower than other two cases, which might be due to the significant activation of pyruvate kinase by low ATP level under extremely low oxygen supply. This may be confirmed by the calculated fluxes via pyruvate kinase as shown in Fig. 6. Cell growth rate and lysine production rate were then decreased to some extent partially due to the decrease of anaplerotic fluxes in this case.

### 3.2.2. Intracellular metabolic reactions

Rich metabolic information may be extracted from intracellular flux distributions evaluated based on the metabolic network model and the measured experimental data. Phase II was chosen for comparison of fermentation characteristics among different DO concentrations, since this phase is the important phase for lysine production, as well as certain cell growth and byproducts formation. To simplify the comparison, the averaged flux distributions (synthesized by the fluxes of two stages) of this phase were used without distinguishing the two different stages. The simplified metabolic maps with the fluxes calculated for phase II are shown in Fig. 7. It is known that the reducing equivalent, NADPH, in the cell plays an important role in biomass synthesis and lysine production since about 4 mol of NADPH is required for 1 mol of lysine formation. In aerobic fermentation, the flux via PP pathway was significantly greater than those of microaerobic cases, and more than 40% of NADPH required was provided by PP pathway in this aerobic case. The NADPH generated through PP pathway decreased with the decrease of oxygen supply due to the cellular regulation of enzymes in PP pathway, since NADPH requirement seems to decrease during cell growth and lysine production phases under oxygen limitation. It should be noted that one of the TCA metabolites, α-ketoglutarate, accumulated significantly in the case where DO concentration was 5%, which may be due to the repressed α-ketoglutarate dehydrogenase complex activity by high intracellular NADH concentration [20], since it was indicated by Zupke et al. [16] that the ratio of $NAD^+$/NADH in the cell might decrease with the decrease of DO concentration in the medium. Despite the significant formation of this byproduct, the flux of PEP carboxylation branch was amplified due to the lowered TCA cycle activity, and the lysine molar yield in this phase was not affected by the byproduct when compared with aerobic fermentation.

Although the TCA cycle activity for the case where DO concentration was 1% tends to be much repressed as compared with the case where DO concentration was 5%, the secretion of α-ketoglutarate was relatively small, which may be due to the fact that large fraction of carbon source channeled to lactate or other byproducts before entering TCA cycle in order to oxidize overproduced NADH. Due to the same reason, the flux of PEP carboxylation decreased, which resulted in the decrease of lysine production.

From phase II to phase III, lysine production rate generally decreases due to the decrease in cell activity, such as the cases of microaerobic culture where DO concentration was 1% and aerobic fermentation. However, for the microaerobic culture where DO concentration was 5%, the lysine yield increased from average value of 0.27 mol $mol^{-1}$ glucose in phase II to 0.31 mol $mol^{-1}$ glucose in phase

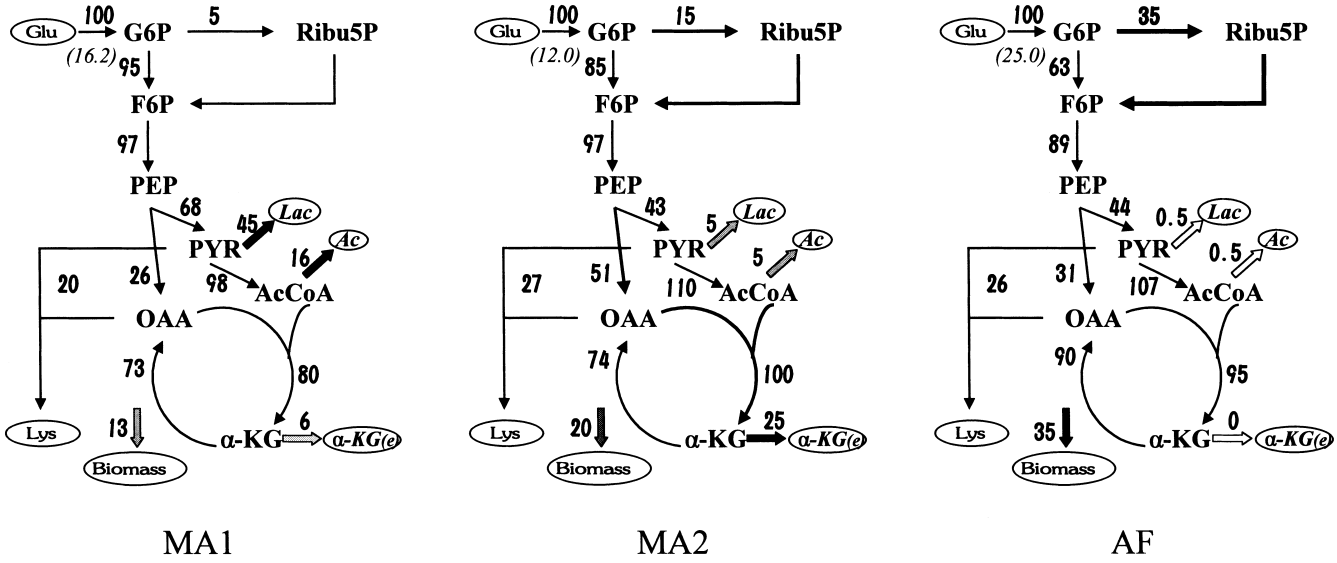


Fig. 7. Comparison of fluxes in phase II under different DO conditions. (Fluxes were normalized by glucose uptake rate, shown in parentheses: mmol $l^{-1}$ $h^{-1}$). MA1: microaerobic culture with 1% DO concentration; MA2: microaerobic culture with 5% DO concentration; AF: aerobic fermentation.

III. Despite the detailed mechanism why this happened is not clear, it was considered that further amplified flux of PEP carboxylation kept the high lysine production rate. Fig. 8 shows the flux change from phase II (average flux) to phase III (estimated at the time interval from 54 h to 55 h) in the case where DO concentration was 5%. More α-ketoglutarate tended to secret in the later phase as compared with phase II, which might be considered to be due to further repression of α-ketoglutarate dehydrogenase complex activity as the intracellular NADH increased at relative high cell concentration [16]. Flux of anaplerotic route increased significantly in response to the large amount of secretion of TCA cycle-related metabolites and at the same time contributed to the lysine secretion. The NADPH requirement in phase III was well allocated and provided by PP pathway and isocitrate dehydrogenation in TCA cycle. Some lactic acid was consumed from phase II to phase III, which may be due to the material and energy requirements, since the flux of TCA cycle became rather low due to the secretion of α-ketoglutarate. In phase III of the case where DO concentration was controlled at 1% saturation, only about 0.1 mol $mol^{-1}$ glucose of lysine yield was observed due to the limited anaplerotic flux since fair amount of lactic acid was still secreted (data not shown).

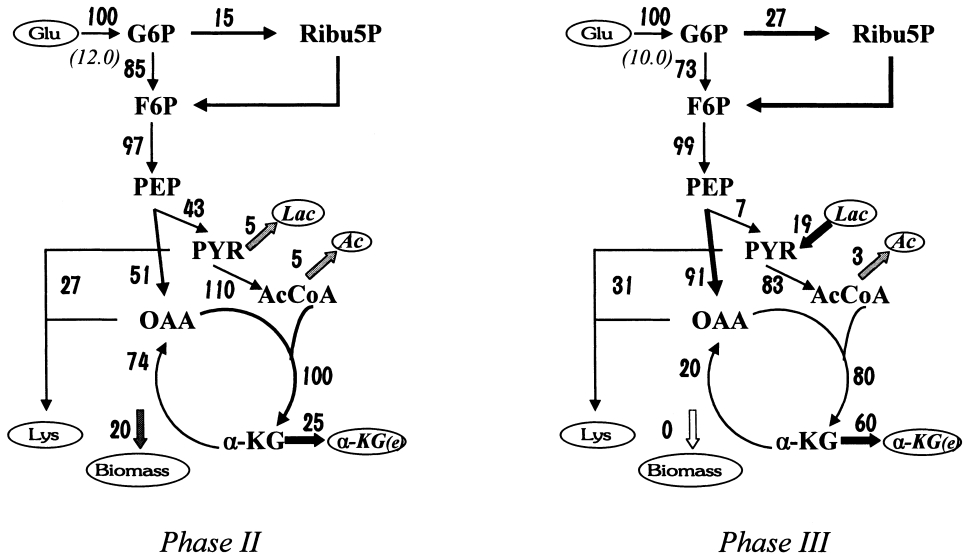


Fig. 8. Comparison of fluxes between phase II and phase III (estimate at the time interval from 54 h to 55 h) of microaerobic culture with 5% DO concentration. (Fluxes were normalized by glucose uptake rate, shown in parentheses: mmol $l^{-1}$ $h^{-1}$).

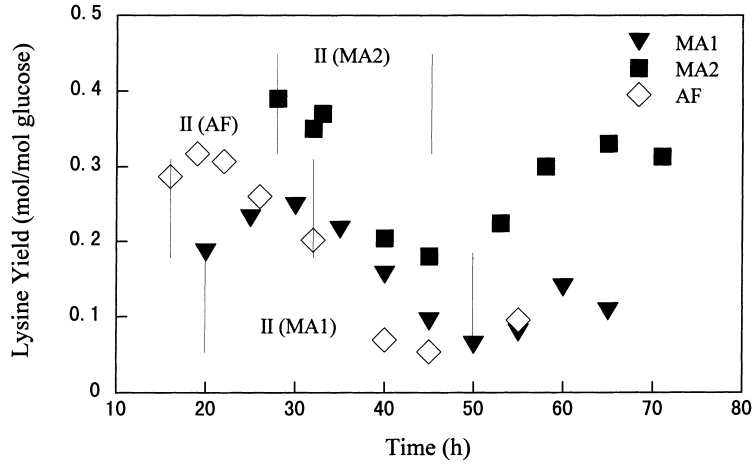


Fig. 9. Local lysine yields of fermentation under different oxygen supply. MA1 (▼): microaerobic culture with 1% DO concentration; MA2 (■): microaerobic culture with 5% DO concentration; AF (◇): aerobic fermentation. II: phase II.

### 3.3. Limiting factors for lysine yield

High local lysine yields could be seen at the early stages of phase II in aerobic and microaerobic fermentation, and at the phase III of the microaerobic cultivation where DO concentration was 5%, where the high yield of about 0.31 mol $mol^{-1}$ glucose could be kept for a long duration (Fig. 9). However, these lysine yields are much less than the theoretical maximum yield of 0.75 mol $mol^{-1}$ glucose, evaluated solely from the metabolic balance constraints [21]. NADPH is known as a key intermediate for cell growth and lysine production, but careful observation for different fermentation or various phases in the culture indicates that the intracellular NADPH may not be the main factor which limits the lysine yield, since enough NADPH could be generated in any case through regulating the fluxes of PP pathway and isocitrate dehydrogenation, which agrees with the flexibility of G6P node as demonstrated by Vallino and Stephanopoulos [22]. In continuous cultures with full oxygen supply, Kiss and Stephanopoulos [11] concluded that the large discrepancy between theoretical and actual yields might be due to high TCA cycle activity coupled with rigid regulation of fluxes at intracellular PEP node. However, the rigidity of this PEP principal node under the perturbation of DO concentration in the medium could not be identified in the present research, since the ratio of PEP carboxylation flux to the flux of PEP synthesis varied markedly with the change of oxygen supply (see Fig. 7). Although in the case of microaerobic culture with 5% DO concentration, the split-ratio for OAA branch at PEP node was rather high (from about 25% to 50%), most OAA converted via PEP carboxylation was unfortunately replenished into TCA cycle rather than the conversion to aspartate as shown in Fig. 10. On the other hand, although more than 70% of PEP carboxylated was converted to aspartate for the microaerobic culture where DO concentration was 1%, the lysine yield was limited low due to the relatively low fraction of OAA branch from PEP node (giving only between 10% and 15%). This low fraction of OAA branch was mainly caused by large amount of inevitable secretion of lactic acid under

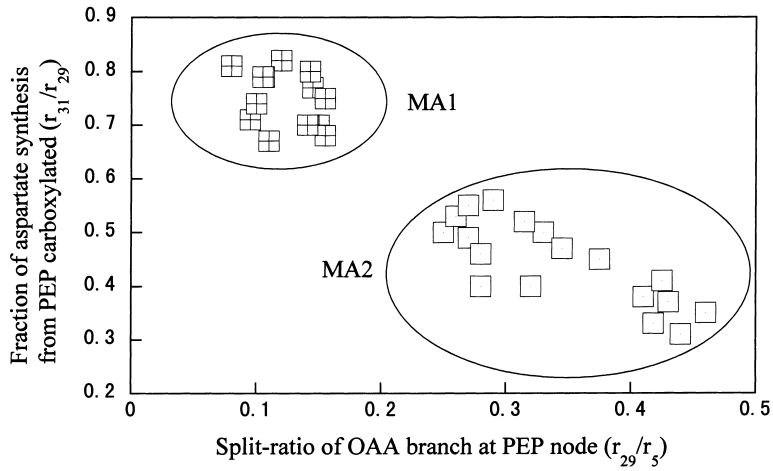


Fig. 10. Distributions of split-ratio of OAA branch at PEP node and the fraction of aspartate synthesis from PEP carboxylated under microaerobic conditions. MA1 (⊞): microaerobic culture with 1% DO concentration; MA2 (□): microaerobic culture with 5% DO concentration.

extreme oxygen limitation. As a consequence, the inconsistency between the split-ratio for PEP carboxylation ($r_{29}/r_5$) and the fraction of aspartate synthesis from PEP carboxylated ($r_{31}/r_{29}$) may be considered as the main yield-limiting factor under microaerobic conditions. In spite of this, the prerequisite of high lysine yield, i.e. high split-ratio for OAA branch at PEP, seems to be attained in microaerobic culture with suitable oxygen supply (such as 5% DO level), and the improvement of lysine yield can then be expected through enhancing activities of some enzymes in lysine synthesis pathway from aspartate under this or similar environmental condition.

### 3.4. Sensitivity consideration

When the extracellular measurement and the metabolic flux model were utilized to estimate the intracellular flux distribution, one should pay more attention to that to what extent the estimated fluxes are affected by the possible errors in extracellular measurements. With the system stoichiometry, the sensitivity of reaction flux vector **r** upon changes on conversion rate vector **q** can be determined as

$$\frac{\partial \mathbf{r}}{\partial \mathbf{q}} = (\mathbf{A}^{\mathrm{T}}\mathbf{A})^{-1}\mathrm{A}^{\mathrm{T}} \tag{3}$$

Here only the effect of extracellular measurement error on intracellular flux estimation was considered. A more meaningful *normalized sensitivity coefficient* is defined as

$$F_j^i = \left(\frac{\partial r_i}{\partial q_j}\right)\left(\frac{q_j}{r_i}\right) \tag{4}$$

where $r_i$ is any flux in **r** and $q_j$ is any extracellular measurement in **q**. Normalized sensitivity calculation (calculated using typical flux values in phase II of culture MA2) indicates that the normalized sensitivities of most intracellular fluxes with respect to glucose consumption rate were relatively high (0.8–1.0), while the sensitivities with respect to other main extracellular conversion rates, the normalized sensitivities of EMP, TCA and anaplerotic fluxes were generally less than 0.1–0.15 except for slightly high sensitivities exhibited with respect to extracellular lysine production rate (0.3–0.4). The calculation of normalized sensitivities indicates that most estimation of intracellular fluxes would be only slightly affected by the error in most extracellular measurements except for glucose and lysine measurements, to which particular attention should be paid.

## 4. Conclusion

In the present study, microaerobic lysine fermentation using *C. glutamicum* ATCC 21253 was carried out under different oxygen supply conditions, and the metabolism in the cell was analyzed with the estimated intracellular flux distributions. The important fermentative characteristics under microaerobic condition is the lysine production capability in the later culture phase. The decrease of TCA cycle enzymes activities under microaerobic conditions may be the main reason why the lysine secretion occurred in the later phase of fermentation. In the present case, the decreased α-ketoglutarate dehydrogenase complex activity might have led to the amplified PPC flux for the case where DO concentration was 5%, which then partly contributed to the lysine secretion. In contrast, only small amount of lysine production was observed in the later phase of aerobic fermentation due to the excess activation of TCA cycle in that phase, as confirmed by the large flux value of TCA cycle calculated in this study (data not shown) or those demonstrated by other researchers [11]. Furthermore, two different metabolic fates could be observed for the different microaerobic conditions, i.e. large amount of lactic acid secretion with low anaplerotic flux and secretion of TCA metabolites with relatively high PEP carboxylation rate corresponding to different situations of oxygen supply. For these two kinds of microaerobic cultures, relatively low flux of anaplerotic route or aspartate synthesis from OAA limited lysine production. Although most of the OAA carboxylated from PEP had to enter TCA cycle in the case where DO concentration was 5% due to the secretion of undesired TCA metabolite, this environmental condition may be employed for enhancing lysine yield, since high flux via PEP carboxylase may be attained under this kind of microaerobic condition. It should also be noted that in the present analysis using metabolic flux method, little attention was paid to the intermediates or enzymes in lysine synthesis pathway from aspartate. The lysine synthesis pathway exhibits the comparable importance as anaplerotic route and TCA cycle for lysine secretion. The investigation of this pathway is now in progress in our laboratory. The combination of genetic approaches for modifying the enzymes in TCA cycle or synthetic pathway, as made by several researchers [23–25], and the engineering approach for extending lysine production phase under suitable oxygen supply condition may offer further improvement in lysine yield.

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