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Roles of Glucose and Acetate as Carbon Sources in L-Histidine Production with *Brevibacterium flavum* FERM1564 Revealed by Metabolic Flux Analysis

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Abstract The metabolic flux pattern for L-histidine production was analyzed when glucose and/or acetate were used as carbon sources. Total L-histidine production was enhanced when mixed substrate (glucose and acetate) was used, compared with that when either glucose or acetate was used as the sole carbon source. Theoretical maximum carbon fluxes through the main pathways for L-histidine production, cell growth, and ATP consumption for cell maintenance were obtained by the linear programming (LP) method. By comparison of the theoretical maximum carbon fluxes with actual ones, it was found that a large amount of glucose was actually used for maintenance of cell viability. On the other hand, acetate was used for cell growth. After depletion of acetate in the mixed substrate culture, the flux for glucose to L-histidine synthesis was markedly enhanced. A strategy for effective L-histidine production using both carbon sources was proposed.

Keywords: L-histidine, metabolic engineering, capability analysis, linear programming

INTRODUCTION

L-Histidine is produced industrially by mutants of *Corynebacterium glutamicum* and *Brevibactrium flavum* from glucose or molasses. Approximately 100 to 1,000 tons of L-histidine is produced annually worldwide for use as a raw material for pharmaceutical derivatives [1]. In order to enhance L-histidine production, strategies of strain improvement by genetic manipulation and optimization of processes operation in fermentation process have been studied. The gene of ATP phosphoribosyl transferase (PRT) was cloned and L-histidine production by the recombinant *C. glutamicum* was improved [2]. Fed-batch cultivation of *B. flavum* FERM1564 and its optimization by boundary control of specific growth rate were developed [3].

It has been reported that when both glucose and acetate were used as the mixed carbon source, a high productivity of L-histidine was realized, compared with that when each carbon source was supplied individually [4]. Metabolic flux distribution analysis using a ^{13}C labeled compound was also reported [5,6] and the important roles of glyoxylate shunt and anaplerotic pathway were studied when acetate was used. However, it remains unclear how both carbon sources were finally utilized for the main pathways of cell growth, maintenance of cell viability, and L-histidine production.

The aim of this study is to investigate the roles of carbon sources, glucose and acetate, in the metabolic pathway based on metabolic flux distribution. Metabolic flux distribution was analyzed off-line [7] and on-line [8] in amino acid fermentation processes overdetermined condition where the sum of the number of measurement variables and stoichiometric equations is the same as or exceeds the number of unknown fluxes in the metabolic network. The information obtained was used for metabolic control analysis [9] and process operation [8]. A method for investigating cell capability was also developed [10-12] under undetermined conditions where the sum of the number of measurement variables and stoichiometric equations is less than the number of unknown fluxes. The capability of *Escherichia coli* to convert some carbon sources, such as glucose, glycerol and acetate, into amino acids and nucleotides was investigated in a metabolic reaction model of *E. coli* by the linear programming (LP) method [10]. The change in metabolic flux distribution was also investigated when environmental conditions, such as oxygen supply [11] and carbon source [12], were changed among several fermentation processes. The change in mole flux distribution was also predicted when some of the genes in the pathway were deleted. It is expected that genomics and bioinformatics data will be integrated for the construction of a map to be analyzed [13,14].

In this study, cell growth and L-histidine production using glucose, acetate, or both carbon sources were compared. First, a metabolic reaction (MR) model based on the stoichiometric balance was developed. Second, metabolic flux distribution was analyzed under several

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cultivation conditions and it was investigated why the simultaneous uptake of two substrates (glucose and acetate) enhanced L-histidine production. Finally, the roles of the two carbon sources were studied through comparison of the theoretical maximum fluxes obtained by the LP method with experimental ones.

MATERIAL AND METHODS

Microorganism and Medium

A key enzyme in the L-histidine synthetic pathway, phosphoribosyl-pyrophosphate (PRPP) synthetase, is repressed by uridine nucleotide produced by uracil [3]. Thus, a uracil auxotroph mutant strain, *B. flavum* FERM1564, was developed for use in the L-histidine production in this study. *B. flavum* FERM1564 was given from National Institute of Bioscience and Human Technology, Japan, and the parent strain of this L-histidine producing mutant was *B. flavum* JCM 1308 or ATCC14067 [3]. The initial concentration of uracil in the batch culture was set at 100 mg/L so that it would not affect either the cell growth or L-histidine production. A synthetic medium with the following composition was used for preculture (per liter of deionized water): 50 g glucose, 10 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 5H_2O$, 2 mg $FeSO_4 \cdot 7H_2O$, 2 mg $MnSO_4 \cdot 4H_2O$, 2 mg biotin, 2 mg thiamine. pH and temperature were adjusted to 7.4 and 30°C, respectively.

Cultivation Conditions for Main Culture

The same medium as that used in preculture was used for the main culture, but glucose or acetate was used as the sole carbon source or as the mixed carbon source. The main cultivation was carried out in the batch mode. A 5-L jar fermenter (MDL-500, MB-Marubishi, Japan) with a liquid working volume of 3.0 L was used. The conditions of pH and temperature were the same as those of preculture. pH was controlled by the addition of 28 wt% ammonia. Temperature, DO, pH, air flow rate, concentrations of CO_2 and O_2 in the exhaust gas, and cell concentration were measured online and the data were transferred to a personal computer (PC-286, Epson, Japan) through a data logger (Themodac-E, Etoh Electric Co., Japan) every minute. Noise in the measured variables was removed using the first-order time delay filter and/or the extended Kalman filter. The details of the procedure were described previously [8]. 50 g/L glucose and/or 5 g/L acetate were used as carbon sources. Cultures on the sole carbon source and the mixed carbon source were called hereafter as glucose culture, acetate culture, and mixed substrate culture, respectively.

Off-line Analysis

Cell concentration was measured in terms of optical density by a UV spectrophotometer (U-2000, Hitachi Co. Ltd., Japan). Glucose concentration was measured by a glucose sensor (Model-2700, YSI Inc., USA). Acetate concentration was measured enzymatically (F-kit acetate, Roche Diagnostics, Japan). Amino acid concentration was measured by an amino acid analyzer (LC model 1, Waters Chromatography Div., Japan). Uracil concentration was measured by HPLC (L-4000, Hitachi, Japan).

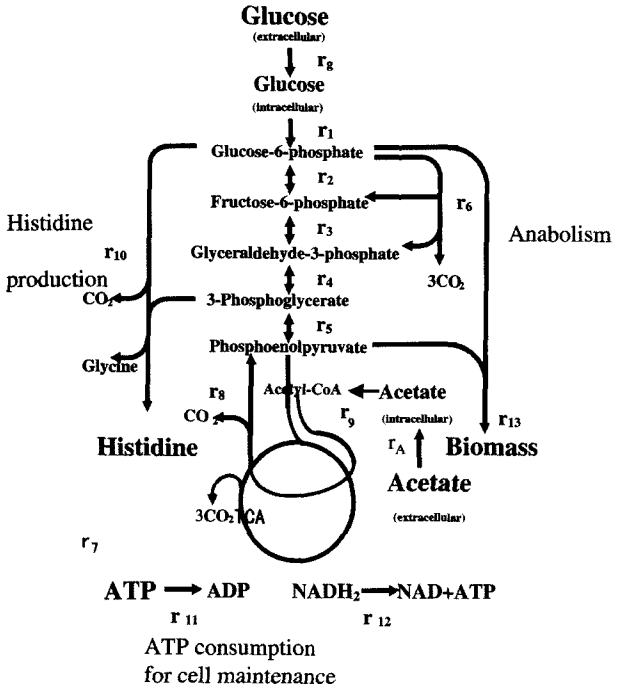

Fig. 1. Simplified model of histidine production by *B. flavum*.

Construction of Metabolic Reaction Model

A simple metabolic reaction model of L-histidine production was constructed. A total of sixty biochemical reactions (not shown) were taken into account. The method developed by Mavrovouniotis and Stephanopoulos [15] was used for simplification of the model and finally, a simplified model with thirteen stoichiometric balance equations was constructed as shown in Fig. 1. The stoichiometric equations are summarized as follows.

In this model, the reactions involved in glucose uptake (r_1 corresponds to mole uptake rate of glucose), glycolysis (r_2-r_5), pentose phosphate pathway (r_6: 3Glc6P => Frc6P + GAP + $3CO_2$ + 6NADPH), TCA cycle (r_7: PEP => $3CO_2$ + 2ATP + 14/3NADH), glyoxylate cycle and anaplerotic pathway (r_8: 2AcCoA => PEP + CO_2 + 8/3NADH), acetate uptake (r_9 corresponds to mole uptake rate of acetate), L-histidine synthesis (r_{10} corresponds to mole production rate of L-histidine: GLc6P + 3-PG + 6ATP + $4NH_3$ => His + Gly + CO_2 + 3NADH), excess ATP (ATP consumption for cell maintenance (r_{11})), oxidative phosphorylation (r_{12}), anabolism (r_{13} corresponds to mole growth rate: aGluc6P +

bPEP + NH_3 + $(MW_x/Y_{ATP})ATP$ + NADPH => $C_{4.88}H_{8.41}O_{2.302}N$ (biomass)) were included, where parameters a, b, c, cell molecular weight, MW_x, cell yield with respect to ATP, Y_{ATP}, and P/O ratio in oxidative phosphorylation, (P/O), were constant.

Mole Flux Distribution Analysis with Fermentation Data

Mole fluxes of r_i (i=1...13) were determined with seven measurements of the reaction rates of cell growth, glucose consumption, acetate consumption, O_2 consumption, CO_2 production, L-histidine production, and L-glycine production. It was also assumed that the concentrations of nine intracellular metabolites, namely, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), glyceraldehyde-3-phosphate (GAP), 3-phosphoglycerate (G3P), phosphoenolpyruvate (PEP), acetyl coenzyme A (AcCoA), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NADH), and nicotine amide adenine dinucleotide phosphate (NADPH) were constant. Since the information redundancy was three, namely, the sum of the number of observed variables (seven) and the number of balance equations of intracellular metabolites (nine) was greater than the number of unknown fluxes (thirteen) by three, the reaction rates were determined by the pseudo-inverse (the least squares form) method [8].

Theoretical Maximum Mole Flux Distribution Analysis

Theoretical maximum mole fluxes based on the MR model were obtained by LP method. The stoichiometric equation is underdetermined [10] since the number of unknown fluxes exceeds the constraints and known fluxes (we did not use any other measurement variables except glucose and acetate uptake rates in this analysis). Mole fluxes to maximize the objective function were determined by the LP method. In this study, three objectives, that is, cell growth, L-histidine production, and ATP consumption for cell maintenance, were aimed at. For the stochiometric equation that is composed of all the reversible reactions, fluxes of forward and backward reactions were defined individually because only the positive values of fluxes can be treated by the LP method. Theoretically obtained fluxes of glucose, acetate, and mixed substrate cultures were analyzed. Calculation was done by a software package in MATLAB (ver. 5.1, Mathworks Inc., USA).

RESULTS AND DISCUSSION

Metabolic Flux Analysis of L-Histidine Production

Fig. 2 shows time courses of fermentation on mixed substrate (both glucose and acetate as carbon sources). The initial concentrations of glucose and sodium acetate in the mixed substrate culture were 50 g/L and 5 g/L, respectively. Uracil concentration was initially 100 mg/L. It was observed that this strain consumed glucose, acetate, and uracil, simultaneously. Although L-histidine was slightly consumed before the depletion of acetate, it was produced again after the depletion of acetate. After the depletion of uracil, cell growth almost stopped and only L-histidine was produced. According to these observations, the fermentation phases were classified into four: growth and L-histidine production using glucose and acetate as carbon sources (phase I), depletion of acetate (phase II), growth and L-histidine production using glucose as the sole carbon source (phase III), L-histidine production on glucose after uracil depletion (phase IV). Start of phase IV was not clear due to long sampling interval of uracil measurement, which might have started at 36 h.

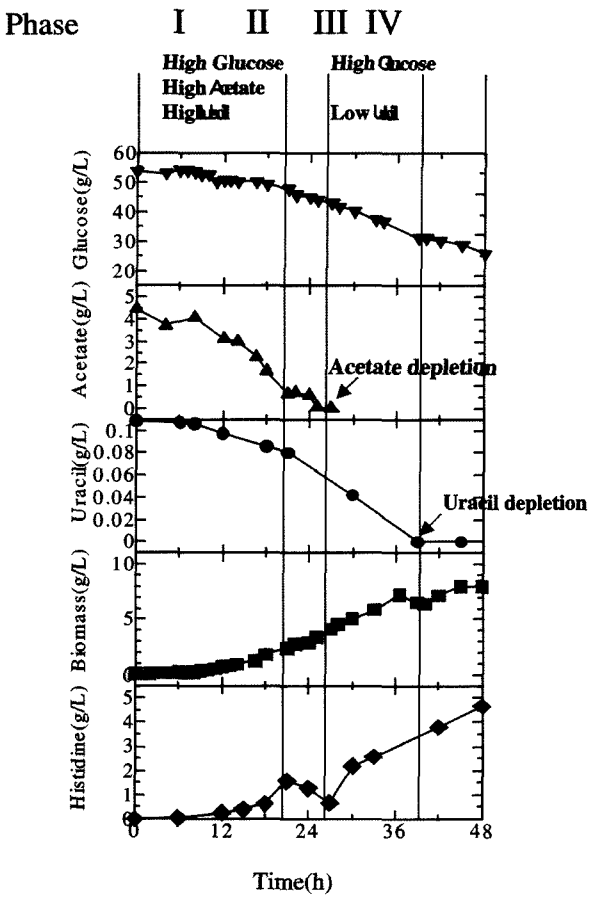

Fig. 2. Time course of L-histidine production in mixed substrate (glucose and acetate) culture.

Fig. 3 shows the time courses of fermentation using glucose (3(a)) and acetate (3(b)) as the sole carbon source. The initial concentrations of glucose (Fig. 3(a)) and acetate (Fig. 3(b)) were the same as those of the mixed substrate culture of 50 g/L and 5 g/L, respectively. Uracil concentration was also the same as that of the mixed substrate culture. By comparing Figs. 3(a) and 3(b), all the reaction rates in Fig. 3(a) were higher than

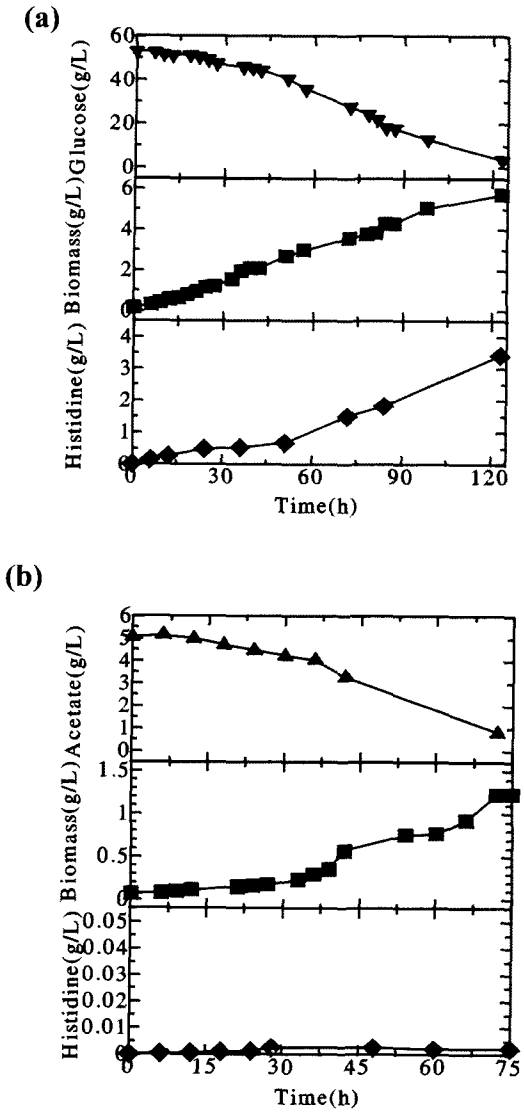

Fig. 3. Time course of L-histidine production in glucose culture (a) and acetate culture (b).

those in Fig. 3(b). However, in Fig. 3(a), the reaction rates for cell growth and L-histidine production were much lower than those in the mixed substrate culture by comparing the time courses of fermentation in Fig. 3(a) with those in Fig. 2. More importantly, when acetate was the sole carbon source, L-histidine production was very low but cell yield with respect to acetate was as high as that in the mixed substrate culture.

Metabolic flux analyses in three fermentations, mixed substrate culture, glucose culture, and acetate culture, are shown in Figs. 4(a), 4(b), and 4(c), respectively. Metabolic fluxes for L-histidine synthesis, cell growth, and ATP consumption are indicated in large font with underline. These reaction rates show how glucose and acetate are finally used in the metabolic pathways. Other reaction rates are indicated in normal font size. In Fig. 4(a), the flux distributions in phases I and III are

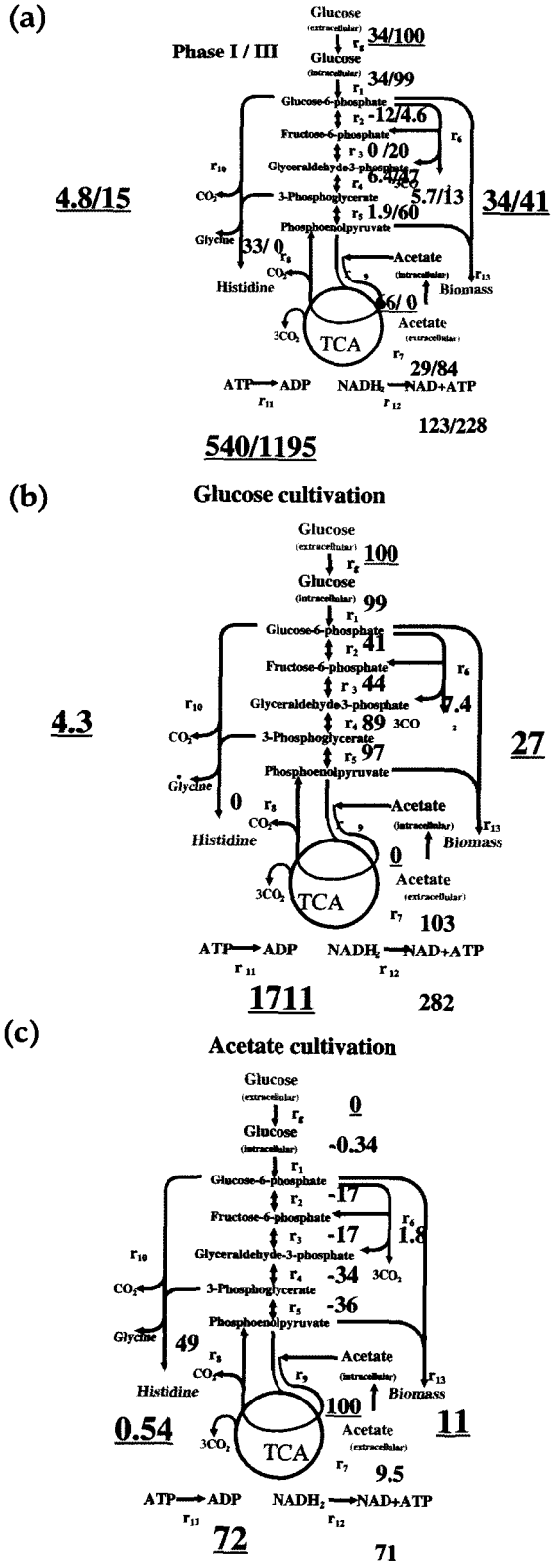

Fig. 4. Metabolic flux distribution in mixed substrate culture (Phase I/Phase III) (a), glucose culture (b), and acetate culture (c). Numbers indicate mole fluxes of metabolic pathways, which are normalized by the total consumption rate of glucose and acetate as 100.

shown. It was found that the flux for L-histidine synthesis in phase III was higher than that in phase I. When glucose was used as the sole carbon source (Fig. 4(b)), a large amount of carbon was oxidized in the TCA cycle and the glyoxylate cycle was not used. Lower flux for L-histidine synthesis and greater ATP consumption for cell maintenance were observed than those in phase III of the mixed culture. In contrast, when acetate was used as the sole carbon source, the glyoxylate cycle and the anaplerotic pathway were enhanced for cell growth. These results were very similar to those of the ^{13}C-labeled compound analysis of *Corynebcaterium glutamicum* [6]. Enzyme activities of isocitrate lyase (ICL) and malate synthase (MS) in glyoxylate shunt of this strain were measured [16]. Specific activities of ICL and MS in acetate culture were about 60 fold and 470 fold greater than those in glucose culture, respectively. Specific activities of ICL and MS in mixed culture were 22 fold and 180 fold greater than those in glucose culture, respectively. These results supported our flux analysis.

It was found that all the mole fluxes became low but the flux for cell formation was relatively high in this culture when the fluxes were normalized by the acetate consumption rate and the L-histidine synthetic flux was low. These flux distributions explained the time courses of fermentation shown in Figs. 2, 3(a) and 3(b). The roles of acetate and glucose in L-histidine production will be discussed later.

Analysis of Cell Capability for L-Histidine Production

The theoretical maximum fluxes for L-histidine production, cell growth, and ATP consumption for cell maintenance were determined by the LP method. The results are summarized in Table 1. When glucose was the sole carbon source, the theoretical maximum flux for L-histidine was calculated as 66.7 (mol %). This is due to the fact that one molecule of L-glycine (C2 compound) and one molecule of CO_2 were required for the production of one molecule of L-histidine from glucose. Regarding the regeneration of redox power, although one mole of NADPH was required for L-histidine synthesis, NADPH was regenerated in the pentose phosphate pathway, and was involved in the pathway from G6P to L-histidine. Therefore, no extra carbon consumption was necessary for NADPH regeneration.

In order to maximize cell growth, carbon flux for the TCA cycle and cell formation were increased to maximum levels with neither L-histidine production nor ATP consumption for cell maintenance. In this case, cellular yield of glucose was 81 C-mol%. Glucose at 19 C-mol% was released as CO_2 in the reaction of pyruvate dehydrogenase complex and the TCA cycle (r_7) because the ratio of C, H, O, and N in the biomass is 4.88:8.41:3.02:1 and mole flux ratio of the glucose uptake to maximum cell formation was 99.5%. 81% (4.88/6) of carbon in glucose should be found in the biomass and 19% of carbon should be released as CO_2. In the calculation of the maximum consumption rate of ATP, all the glucose was oxidized in the glycolytic pathway and the TCA cycle with neither L-histidine production nor cell growth.

Table 1. Theoretical maximum fluxes for main pathways (mole %) r_i^{max}

Fluxes were normalized by assigning carbon source uptake flux as 100

	L-histidine (r_{10})	Biomass (r_{13})	$m_{ATP}(r_{11})$
Glucose culture	66.7	99.5	3,800
Acetate culture	16.7	19	1,100

When acetate was used as the sole carbon source, the maximum flux of L-histidine was decreased (16.7 mol %) because of two reasons: (1) two carbon atoms comprised one molecule of acetate, which was one-third of the number of carbon atoms comprising glucose. (2) Carbon flux from acetate to L-histidine should take place via the anaplerotic pathway from oxaloacetate (4C compound) to phosphoenolpyruvate (3C compound). One molecule of CO_2 production should be produced according to this reaction, that is, 1/4 of the total amount of carbon atoms was released in this reaction in addition to the carbon flux from G6P to the L-histidine synthetic pathway. From these considerations, it was found that glucose was a better carbon source than acetate from the viewpoint of production yield.

When mixed substrates were used, the maximum yield was expressed as a linear combination of the maximum yield using glucose and the maximum yield using acetate with the ratio of mixed substrates. For example, when 30% glucose and 70% acetate were used, the flux distribution for maximum cell growth was given by a linear combination of theoretical flux distributions for each carbon source with weights of 30% and 70%, respectively. In conclusion, the theoretical maximum yield of L-histidine was obtained when only glucose was used. However, L-histidine production using the mixed substrates was higher than that using a sole carbon source. In the next section, why the mixed substrates led to the maximum production of L-histidine in the actual fermentation is discussed by comparing actual flux distributions with the theoretical one.

Comparison of Actual Flux Distributions with Theoretical Distributions

Actual flux distributions in glucose culture and acetate culture were compared with theoretical ones obtained by cell capability analysis. Table 2 shows the ratio of mole fluxes estimated from experimental data to the theoretical maximum fluxes for L-histidine, biomass, and m_{ATP}. In glucose and acetate cultures, carbon recovery was low to moderate (70-80%). This suggested that the production of other metabolites such as fatty acids and amino acids, and/or errors of on-line and off-line measurement were involved. Particularly, carbon recov-

Table 2. Ratio of actual to theoretical flux distributions in the sole carbon source culture (mol%)

	L-Histidine (r_{10})	Biomass (r_{13})	m_{ATP} (r_{11})	Carbon recovery
Glucose (Sole C source)	6.4[a] (8.2[b])	27 (34)	45 (57)	78 (100)
Acetate (Sole C source)	3.2 (4.7)	57 (85)	6.5 (9.7)	67 (100)
Glucose (Mixed C sources (Phase III))	24 (25)	42 (43)	31 (32)	97 (100)

[a] values indicate percentage of mole flux estimated from experimental data.
[b] values indicate relative percentage flux for each pathway (f_i).

ery in acetate culture was low. However, in phase III of mixed carbon source, carbon recovery is 97% and the reaction model represented the metabolic flux distribution in each phase of fermentation well.

In order to investigate the roles of the main pathways for L-histidine synthesis, biomass, and m_{ATP}, the relative percentage among these three pathways was calculated and results are shown in parentheses in Table 2. In this analysis, the actual flux distribution was determined by superposition with the weighting coefficients of each original flux distribution for maximum L-histidine production, maximum cell growth, and maximum ATP consumption for cell maintenance, respectively. For example, when glucose was used as the sole carbon source, the superposition of the flux distribution of the theoretical maximum L-histidine production with 8.2%, that of the theoretical maximum cell growth with 34%, and that of the theoretical maximum ATP consumption with 57%, gave that actual flux distribution in Fig. 4(b), where the ratio of actual L-histidine production to the theoretical maximum one, that of actual cell growth to the theoretical one, and that of actual ATP consumption to the theoretical one are 8.2, 34, and 57%, respectively. Thus, it is interpreted that a large amount of glucose (57%) was necessary for cell maintenance in glucose culture. That is, the calculated weighting coefficients indicated how much percentage of carbon source was actually used in each main pathway.

Table 2 summarizes the above results. Much acetate (85%) was used for biomass formation in the acetate culture. Furthermore, after the depletion of acetate in the mixed substrate culture (phase III), the ratio of flux for cell maintenance was lower (32%) than that in the glucose culture and much glucose (25%) was used for L-histidine production. It was found that the ATP consumption for cell maintenance was decreased and high L-histidine production was realized when both carbon sources were used.

Evaluation of Role of Glucose and Acetate in Mixed Substrate Culture

As has been described above, actual flux distribution can be expressed as the superposition of theoretical maximum flux distribution with weighting coefficients. In other words, the roles of carbon sources can be evaluated based on these weighting coefficients. To determine the role of glucose and acetate in the mixed substrate culture, three main mole fluxes were calculated as

Table 3. Comparison of fluxes calculated by linear combination of sole carbon source culture with experimental data

	Estimation	Experiment
r_{His}	2.4	4.8
r_X	22	34
r_{mATP}	651	540

$$r_{His} = r^{max}_{His/Gluc} \cdot \frac{f_{His/Gluc}}{100} + r^{max}_{His/Ac} \cdot \frac{f_{His/Gluc}}{100} \quad (1)$$

$$r_X = r^{max}_{X/Gluc} \cdot \frac{f_{X/Gluc}}{100} + r^{max}_{X/Ac} \frac{f_{X/Ac}}{100} \quad (2)$$

$$r_{mATP} = r^{max}_{mATP/Gluc} \frac{f_{mATP/Gluc}}{100} + r^{max}_{mATP/Gluc} \frac{f_{mATP/Ac}}{100} \quad (3)$$

where r_i, r_i^{max}, f_i, are mole flux for the mixed substrate culture, the maximum mole flux for the sole carbon source culture, percentage of mole flux for each pathway in the sole carbon source culture, respectively. The calculation results were compared with the results of flux analysis in Fig. 4(a) (phase I) in Table 3. Some differences between the calculated values and the experimental ones were observed. However, the mole fluxes for the different pathways showed a similar magnitude. This findings suggested that the roles of glucose and acetate in mixed substrate culture (phase I) were basically the same as those in the sole carbon source culture

Strategy for Optimal L-Histidine Production

As discussed above, glucose is a good carbon source for L-histidine synthesis because a small amount of carbon was released in the synthetic pathway of L-histidine (high yield). On the other hand, it was found that acetate was effectively used for cell growth because the ratio of actual biomass flux to the theoretical one reached 85%, as shown in Table 2. However, the specific growth rate using acetate was much lower (0.039 h^{-1}) than that of phase I of the mixed substrate culture (0.23 h^{-1}). The ratio of actual flux for L-histidine production to the theoretical one in phase III was the highest (25%) and the specific production rate was also the highest (0.026 g-his(g-cell)$^{-1}$ h^{-1}), compared with those in glucose culture (4.1×10^{-3} g-his(g-cell)$^{-1}$ h^{-1}) and acetate culture (4.5×10^{-3} g-his(g-cell)$^{-1}$ h^{-1}), respectively.

Finally, an optimal production strategy was proposed as follows: Use glucose and acetate simultaneously in the growth stage with an appropriate initial concentration of uracil. Stop cell growth by depletion of uracil and enhance L-histidine production. In this phase, glucose should be used as the sole carbon source. This strategy was very similar to a previously reported strategy for boundary control of the specific growth rate [4]. However, the main difference was the mole ratio of glucose to acetate, which was set constant throughout the fermentation in the previous study. However, in this study, it was found that acetate was important for cell growth and glucose after depletion of acetate was effectively used for L-histidine production. Therefore, the combined use of mixed carbon sources and the sole carbon source was proposed as the strategy for optimal L-histidine production.

CONCLUSION

In this study, roles of glucose and acetate as carbon sources in L-histidine production was investigated based on the metabolic flux analysis. Metabolic flux distribution was analyzed under several cultivation conditions and it. It was found that the glucose was used for energy formation for maintenance of cell viability and acetate was used for cell growth in the mixed substrate culture. After depletion of acetate, flux from glucose to L-histidine was markedly enhanced.

NOMENCLATURE

r_i : mole flux (i=1..13) (mole/h)
r_i^{max} : theoretical maximum flux (mole/h)
f_i : percentage of mole flux in three main pathways (%)

Suffix

His/Gluc : histidine synthesis from glucose
X/Gluc : biomass synthesis from glucose
m_{ATP}/Gluc : ATP consumption for cell maintenance from glucose
His/Ac : histidine synthesis from acetate
X/Ac : biomass synthesis from acetate
m_{ATP}/Ac : ATP consumption for cell maintenance from acetate

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