Journal of Microbiology and Biotechnology
Vol. 4, No. 2, 95-101, 1994

Effect of Glycine on L-Ornithine Production by a Citrulline Auxotroph of *Brevibacterium ketoglutamicum* and Stoichiometric Analysis

NAM, SOO WAN, DAE KEON CHOI, WUK SANG RYU, HYUNG WOOK JANG, BONG HYUN CHUNG AND YOUNG HOON PARK*

Genetic Engineering Research Institute, KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea

The effects of glycine on cell growth and L-ornithine production were investigated in shake-flask and jar fermentor cultures of a citrulline auxotrophic mutant, *Brevibacterium ketoglutamicum* BK 1046. In the shake-flask culture, the optimal concentration of glycine for L-ornithine production was found to be 20 g/l. In the jar fermentor culture with the glycine at an initial concentration of 20 g/l, L-ornithine production increased by 28%, compared to that of the culture with no glycine added. 37 g/l of L-ornithine was produced when additional feeding of glycine (5 g/l) was made. This was a significant improvement in L-ornithine production compared to that (ca. 24 g/l) of the corresponding batch culture conducted without glycine. According to the stoichiometric analysis with the batch fermentation results, the experimental and theoretical L-ornithine yields based on the glucose consumption were 0.24 and 0.59, respectively. This indicates that the performance of L-ornithine fermentation can further be improved by the supplementation of glycine and the development of a mutant strain possessing a higher growth yield.

L-Ornithine is a basic amino acid and also an intermediate metabolite involved in the arginine biosynthesis. It is well known that L-ornithine plays an important role in the mechanism of ammonium detoxification through the "urea cycle" in mammalian. Due to these biological activities, it is being used as a therapeutic agent for the liver. So far, a variety of methods such as the enzymatic decomposition of L-arginine (27) and microbial fermentation (8, 25, 26) have been developed to produce L-ornithine. The first microbial fermentation of L-ornithine was attempted with a L-citrulline auxotrophic mutant of *Corynebacterium glutamicum* by Kinoshita *et al.* (8). The molar yield reached as high as 36% from the glucose in the medium supplemented with a limited amount of L-arginine after 3 days of culture. L-arginine or L-citrulline auxotrophic mutants such as *Brevibacterium lactofermentum, B. kawasaki* and *Arthrobacter citreus* have also been used for L-ornithine production, and the molar yields ranging from 35 to 40% from sugar were achieved with these mutants (13, 23). Further efforts have been made to enhance the molar yield and/or the productivity; *i.e.*, L-ornithine fermentation using hydrocarbons (14, 17, 24), saturated carboxylic acids (32), or precursors (31).

*Corresponding author
Key words: L-ornithine, citrulline auxotroph, *Brevibacterium ketoglutamicum*, glycine, stoichiometric analysis

Glycine is known to be a biosynthetic precursor for the serine, L-ornithine and cell wall, and thus has been used to increase the yield of these amino acids (5, 18-20). In this study, the effect of glycine on L-ornithine production was investigated in shake-flask and fermentor cultures of a L-citrulline auxotroph in more detail. In addition, the stoichiometric analysis on the L-ornithine production was carried out on the basis of the fermentation data, and the experimental yield was compared with the theoretical one. The theoretical analysis offered the potential to further enhance the production of L-ornithine.

BIOCHEMICAL STOICHIOMETRY FOR L-ORNITHINE BIOSYNTHESIS

In this theoretical approach, both the metabolic pathway involved and coenzyme recycling were carefully taken into consideration. When glucose is converted to acetyl-CoA via the EMP pathway, the detailed biosynthetic pathway of L-ornithine from glucose is depicted in Fig. 1 and 2. As, in glutamic acid fermentation, $NADP^+$/NADPH is regenerated by the reactions of isocitrate dehydrogenase and glutamate dehydrogenase, the ratio of $NADP^+$ to NADPH is balanced until glutamate is synthesized (9). Coenzyme A involved in the biosynthesis of acetyl-CoA is self-recycled and balanced

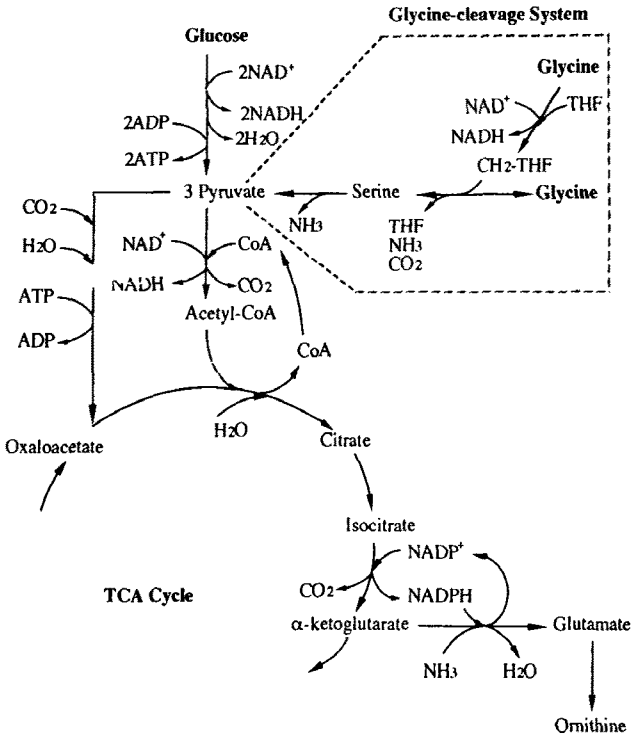

Fig. 1. Biosynthetic pathway of L-ornithine from glucose *via* the EMP pathway and the "glycine-cleavage system".
THF is tetrahydrofolate, and CH_2-THF is methylene-THF.

by the acetyl-CoA reaction and citrate formation. Under the aerobic condition, recyclings of NAD^+/NADH and ATP/ADP are principally achieved by the respiratory chain since the shortage of these coenzymes might prevent a smooth flow of metabolites. From all the biochemical reactions involved in the glutamate biosynthesis (Fig. 1), the resulting net reaction is

$$\text{Glucose}+NH_3+(3/2)O_2 \longrightarrow \text{Glutamate}+CO_2+3H_2O \quad (1)$$

As shown in Fig. 2, the biochemical reaction for the ornithine biosynthesis from glutamate is

$$2\text{Glutamate}+\text{Acetyl-CoA}+\text{ATP}+\text{NADPH}+H^+ \longrightarrow \text{Ornithine}+\text{CoA}+\text{ADP}+\text{Pi}+NADP^+ +\alpha\text{-ketoglutarate} \quad (2)$$

In coryneform bacteria, the majority of N-acetyl glutamate is formed by N-acetylglutamate-acetylornithine acetyltransferase reaction catalyzing the transfer of the acetyl group from acetylornithine to glutamate (28, 29). As in the reaction (1), it can be assumed that recycling of ATP/ADP is achieved by the respiratory chain. Taking into account the reaction of α-ketoglutarate ⟶ glutamate, the net reaction for the ornithine formation from glutamate is

$$\text{Glutamate}+NH_3+2NADPH+2H^+ \longrightarrow \text{Ornithine}+O_2+2NADP^+ \quad (3)$$

Two moles of NADPH are required for the ornithine

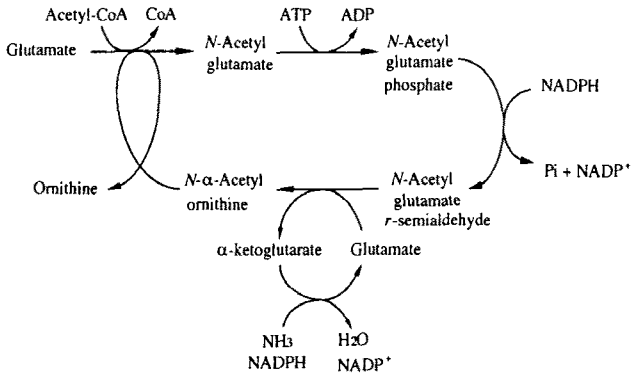

Fig. 2. Biosynthetic pathway of L-ornithine from glutamate.

biosynthesis from glutamate, but the reaction for regenerating NADPH is not found as can be seen in Fig. 1 and 2. Thus, NADPH consumed by reaction (3) should be regenerated by other biochemical reactions for a continuous ornithine biosynthesis. Glucose-6-phosphate and 6-phosphogluconate dehydrogenases in the pentose-phosphate cycle (HMP pathway) are most probable to be involved in the NADPH-regeneration. When 2 moles of NADPH are regenerated via the HMP pathway, the net reaction becomes

$$(1/4)\text{Glucose}+(3/2)O_2+2NADP^+ \longrightarrow (3/2)CO_2+(3/2)H_2O+2NADPH+2H^+ \quad (4)$$

Combining the reactions (1), (3) and (4) yields

$$(5/4)\text{Glucose}+2NH_3+2O_2 \longrightarrow \text{Ornithine}+(5/2)CO_2+(9/2)H_2O \quad (5)$$

Assuming that no biomass forms, the maximum ornithine yield (Y_{p1}*) from glucose (S_1) is

$$Y_{p1}^* = \frac{132.16}{(5/4)\cdot 180} = 0.587 \text{ (g-ornithine/g-glucose)}$$

If the regeneration of $NADP^+$/NADPH is not considered, $Y_{p1}^* = 132.16/180 = 0.734$ (g-ornithine/g-glucose).

The coryneform bacteria possess a "glycine-cleavage system" such as glycine-assimilatory pathway, in which glycine synthase, L-serinehydroxymethyl transferase and serine dehydratase are sequentially involved in the pyruvate formation (1, 7, 11, 12, 18, 19, 21). The detailed "glycine-cleavage system" is shown in Fig. 1, which comprises EMP pathway and TCA cycle. If glycine is added to the culture medium, it could be used as a source of carbon and energy throughout the "glycine-cleavage system". The net reaction for the ornithine biosynthesis from glycine is

$$4\text{Glycine}+(1/2)O_2 \longrightarrow \text{Ornithine}+2NH_3+3CO_2+H_2O \quad (6)$$

In reaction (6), the regeneration of $NADP^+$/NADPH is considered to occur *via* the HMP pathway by using glucose during the cultivation. Assuming that no bio-

mass forms from glycine, the maximum ornithine yield (Y_{p2}*) from glycine (S_2) is

$$Y_{p2}^* = \frac{132.16}{4.75} 0.441 \text{ (g-ornithine/g-glycine)}$$

MATERIALS AND METHODS

Microorganism

The L-citrulline auxotrophic mutant, *Brevibacterium ketoglutamicum* BK 1046, was used in this study. This mutant strain was obtained from *B. ketoglutamicum* ATCC 21092 by UV or NTG treatment. The detailed procedures for the mutation and isolation have previously been described (4).

Culture Media

YPD medium (yeast extract 1%, Bacto-peptone 1%, glucose 2%) was used for the seed culture. The composition of the medium (SM) for the shake-flask culture was as follows (in g/l distilled water): glucose, 40; KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 10; $MgSO_4 \cdot 7H_2O$, 1.0; yeast extract, 1.0; $CaCO_3$, 2; 100 mM trace elements solution (Cu, Fe, Mn, Co, Mo etc), 1 ml. Main fermentation medium (FM) used in the jar fermentor was (g/l distilled water): glucose, 100; KH_2PO_4, 2; $(NH_4)_2SO_4$, 10; $MgSO_4 \cdot 7H_2O$, 1.0; yeast extract, 5; trace elements solution, 1 ml

Culture Conditions

A single colony on the YPD agar plate was inoculated into 10 ml YPD medium and incubated overnight at 30°C. For further activation of the cell, the seed was transferred into 100 ml YPD medium and incubated for 12 hr. 2.5 ml of this second preculture was inoculated into a 500 ml baffled Erlenmeyer flask containing 50 ml of SM, and then the flask was cultivated at 180 rpm for 72 hr. In the jar fermentor (KFC, Korea) operation, the culture conditions were as follows: working volume, 1 l; temperature, 30°C; agitation speed, 600 rpm. The pH was controlled at 7.0 by the addition of 4N NH_4OH.

Analytical Methods

The cell growth was monitored by measuring its optical density (OD) at 600 nm using a spectrophotometer (UVICON 930, Switzeland). The OD value was converted to the dry cell weight (DCW) by using a calibration curve. The glucose concentration was measured using a Glucose and Lactate Analyzer (YSI 2000, USA). L-ornithine was determined colorimetrically after the ninhydrin reaction (3).

RESULTS AND DISCUSSION

Effect of Glycine in Shake-Flask Culture

The effects of glycine on the cell growth and L-ornithine production were investigated in shake-flask cultures containing liquid media of varying concentrations of glycine. When glucose was exhausted after 72 hr of culture, the concentrations of the cell and L-ornithine were measured and compared (Fig. 3). The cell growth was significantly inhibited by glycine when the concentration was above 20 g/l. The addition of glycine at an initial concentration of 40 g/l yielded 1.7 g DCW/l, which corresponded to a half of that obtained without the addition of glycine. L-ornithine production gradually increased with an increasing glycine concentration, but declined rapidly as the concentration was increased above 20 g/l. Less of L-ornithine was produced with media containing glycine at concentrations above 30 g/l than did with a glycine-free medium. This result shows that the glycine at low concentrations was utilized by the cells through the glycine-cleavage pathway, and resulted in a significant increase in the L-ornithine production.

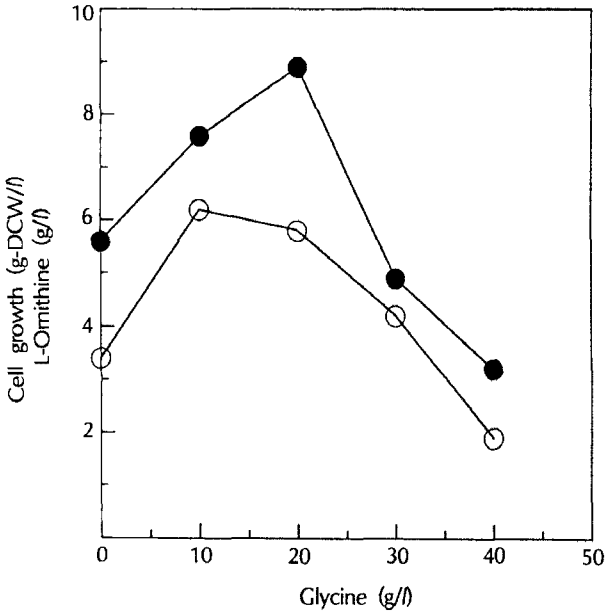

Fig. 3. Effect of glycine on the cell growth and L-ornithine production in the shake-flask culture.
B. ketoglutamicum BK 1046 was cultivated at 30°C and 180 rpm for 72 hr. ○, dry cell weight; ●, L-ornithine concentration.

The excess amount of glycine severely inhibited the cell wall synthesis of *Corynebacterium glutamicum* and resulted in the formation of elongated and swollen cells (15). Furthermore, the cell growth rate decreased by the damages in cell wall functions such as the substrate uptake and resistance to the osmotic pressure. In this study, the bulge cells were also observed at high glycine concentrations (data not shown), and thus the reduced L-ornithine production at glycine concentrations above 30 g/l should result from the damaged function of cell wall. As a result, the optimal concentrations of glycine for the cell growth and L-ornithine

production were found to be 10 g/l and 20 g/l, respectively, from the shake-flask culture experiments.

Effect of Glycine in Jar Fermentor Culture

The glycine of suboptimal concentration of 20 g/l was initially supplemented to a jar fermentor containing the fermentation medium (FM), and the fermentation results were compared to those obtained from the glycine-free FM. To further enhance L-ornithine production, 5 g/l of glycine was additionally fed to the fermentor after 53 hr of culture. As shown in Fig. 4, the cell growth was slightly inhibited at the initial period of fermentation when glycine was added. After 50 hr of culture, however, the cells in the glycine-containing medium grew faster than those in the glycine-free FM, and consequently the final cell density reached 30.6 g DCW/l. The additional feeding of glycine during fermentation resulted in a sudden decrease in cell growth rate, reaching finally 27.8 g DCW/l of cell density.

The patterns of L-ornithine production were much different from those of cell growth, especially after 36 hr of culture. Fermentation with additional feeding of glycine yielded 37 g/l of L-ornithine, which corresponded to an 1.54-fold increase over that of glycine-free medium. The highest volumetric productivity, 0.563 g/l·hr, was also obtained in this fermentation mode (Table 1).

It is of interest to note that L-ornithine was accumulated in the culture broth, even during the period that the cell growth rate was retarded by the additional feeding of glycine. The biosynthesis of peptidoglycan is known to be inhibited by glycine, resulting in a leaky cell wall (5). This property has been used for the transformation of plasmid into the coryneform bacteria (2, 6, 15, 16, 22, 30). Therefore, increased L-ornithine production even with glycine present may be the result of the enhanced permeability of the cell wall as well as more pyruvate concentration formed from glycine.

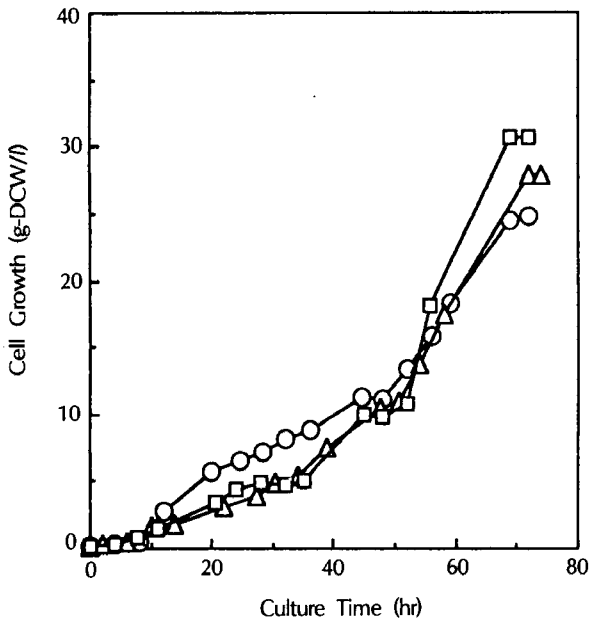

Fig. 4. Effect of glycine on the cell growth in the jar fermentor culture.
O, 0 g/l glycine;□, 20 g/l glycine;△, (20 g/l+5 g/l) glycine.

When glycine is assimilated *via* the "glycine-cleavage pathway", the ammonium ion is formed and consumed as a nitrogen source by the cells. In fermentation with glycine-containing medium, the amount of 4N ammonia solution used for pH control was a half of that with the glycine-free medium (Table 1). This proves that the cells possess a glycine-cleavage system and thus is capable of utilizing glycine as a carbon source by using this system. In consequence, adding glycine at a concentration below 20 g/l improved the L-ornithine production significantly.

Stoichiometric Analysis in the Absence of Glycine

The cellular efficiencies of substrate utilization and product formation were investigated by using the stoichiometric analysis based on the metabolic pathways depicted in Fig. 1 and 2. $-\Delta S_1^{total}$, a total amount of glucose consumed can be divided into two parts; the amount of glucose converted to biomass ($-\Delta S_1^{cell}$), and the amount of glucose converted to L-ornithine ($-\Delta S_1^{omit}$).

$$-\Delta S_1^{total}=(-\Delta S_1^{cell})+(-\Delta S_1^{omit}) \quad (7)$$

The intrinsic cellular yield is defined as $Y_{x1}^*=\Delta X/(-\Delta S_1^{cell})$, and the theoretical L-ornithine yield based on glucose as $Y_{p1}^*=\Delta P_1^*/(-\Delta S_1^{omit})$, where ΔX and ΔP_1 represent the biomass and theoretical L-ornithine concentrations produced, respectively. The theoretical concentration of L-ornithine is given by

$$\Delta P_1^*=\left[(\Delta S_1^{total})-\frac{\Delta X}{Y_{x1}^*}\right]Y_{p1}^* \quad (8)$$

Substitution of the experimental ΔS_1^{total} and ΔX values into eq. (8) makes ΔP_1^* a function of Y_{x1}^*. If

Table 1. Summary of glycine effects on the cell growth and L-ornithine production

	Control	Glycine 2%	Glycine 2%+0.5%
Max. cell mass (g/l)	25.0	30.6	27.8
Max. L-ornithine (g/l)	24	30	37
4 N NH_4OH added (ml)	87	48	43
Max. volumetric productivity (g/l·hr)	0.348	0.435	0.563

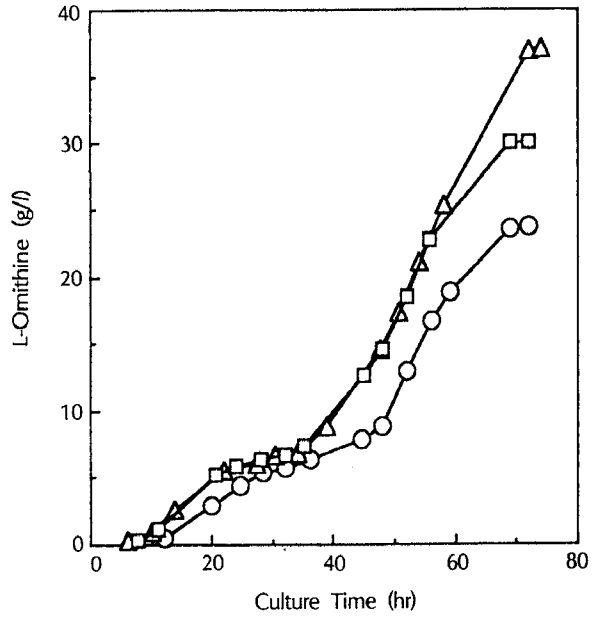

Fig. 5. Effect of glycine on the L-ornithine production in the jar fermentor culture.
Symbols are the same as in Fig. 4.

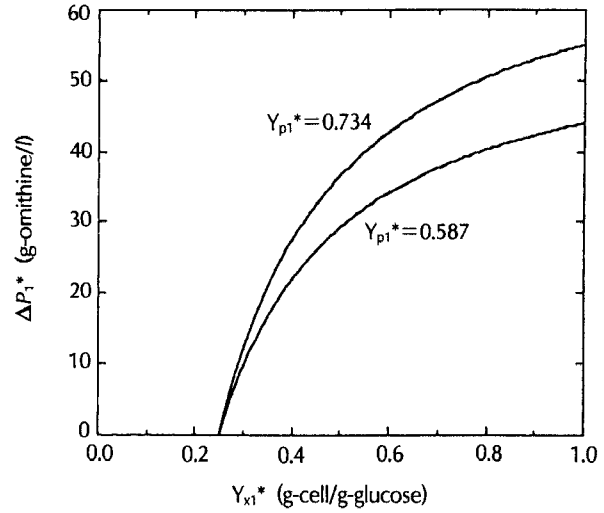

Fig. 6. Theoretical L-ornithine concentration (ΔP_1*) as a function of intrinsic cellular yield (Y_{x1}*) for the cases of which the regeneration of $NADP^+$/NADPH is considered (Y_{p1}*=0.587) or not (Y_{p1}*=0.734).

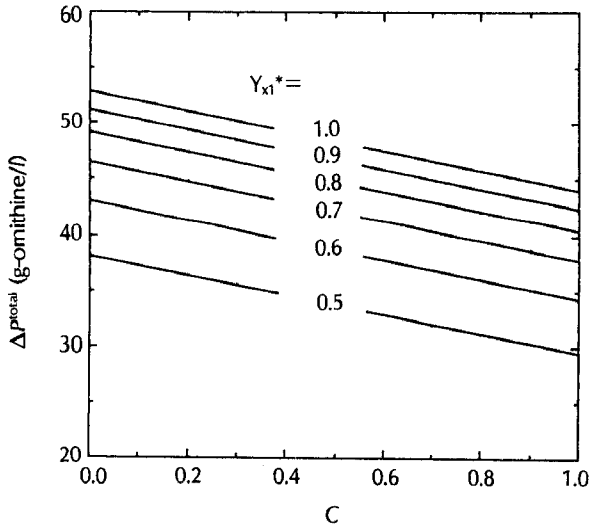

Fig. 7. Theoretical L-ornithine concentration (ΔP^{total}) as functions of conversion ratio (C) and intrinsic cellular yield (Y_{x1}*).

$NADP^+$/NADPH regeneration is neglected, the sensitivity of ΔP_1* to the value of Y_{x1}* might result in the overestimation of ΔP_1* value (Fig. 6). As shown in Fig. 6, the value of ΔP_1* increases with increasing Yx1* value. Theoretically, the value of ΔP_1* can reach up to 40 g/l if a mutant has a cellular yield (Y_{x1}*) over 0.8.

Stoichiometric Analysis in the Presence of Glycine

As mentioned above, glycine can be utilized as a source of both the carbon and energy. Therefore, the total amount of glycine ($-\Delta S_2^{total}$) consumed is divided into two parts.

$$-\Delta S_2^{total}=(-\Delta S_2^{cell})+(-\Delta S_2^{omit}) \quad (9)$$

The theoretical yield of L-ornithine based on glycine consumption, Y_{p2}*, is expressed as ΔP_2*/($-\Delta S_2^{omit}$), where ΔP_2* denotes the theoretical concentration of L-ornithine synthesized from glycine. Therefore, ΔP_2* becomes

$$\Delta P_2^*=(\Delta S_2^{total})\cdot(1-C)\cdot Y_{p2}^* \quad (10)$$

where C represents the conversion ratio of total glycine to the biomass fraction ($C=\Delta S_2^{cell}/\Delta S_2^{total}$). The total concentration of L-ornithine (ΔP^{total}) synthesized from both glucose and glycine is the summation of ΔP_1* and ΔP_2*. Combining the equations (8) and (10) yields

$$\Delta P^{total}=\Delta P_1^*+\Delta P_2^*=\left[(\Delta S_1^{total})-\frac{\Delta X}{Y_{x1}^*}\right]\cdot Y_{p1}^* + (\Delta S_2^{total})\cdot(1-C)\cdot Y_{p2}^* \quad (11)$$

ΔP^{total} becomes a function of C and Y_{x1}* by substituting the fermentation data such as ΔS_1^{total}, ΔS_2^{total}, ΔX, Y_{p1}* and Y_{p2}* into eq. (11). As shown in Fig. 7, the theoretical concentration of L-ornithine increases constantly with decreasing C values, which offers the potential of further increase in L-ornithine production. Theoretically, L-ornithine at a concentration of 47 g/l can be produced at Y_{x1}*=0.8 and C=0.2.

In Table 2, values for the theoretical and experimental yield are summarized, and the actual fermentation results obtained with the glycine-free and glycine-containing media are also compared with the corresponding theoretical ones. The ratio of the experimental yield to the theoretical one was about 0.41 or 0.44, indicating a low cellular efficiency of glucose and glycine for L-ornithine biosynthesis. In the glutamic acid

Table 2. Theoretical yield (Y_p*), theoretical L-ornithine concentration (P*), and experimental yield (Y_p^{exp}) based on the biochemical stoichiometry and fermentation results

Y_p* for glucose	0.587
Y_p* for glycine	0.441
P* from 100 g/l glucose	58.74
P* from 100 g/l glucose and 20 g/l glycine	67.55
Y_p^{exp} from 100 g/l glucose	0.24
Y_p^{exp} from 100 g/l glucose and 20 g/l glycine	0.25
P^{exp}/P* for glucose$^{\xi}$	0.41
P^{exp}/P* for glucose and glycine$^{\xi}$	0.44

$^{\xi}P^{exp}$ is the maximal L-ornithine concentration for the control and 2% glycine in Table 1.

fermentation, the reduction in the product yield was partly caused by the reversibility of malate dehydrogenase reaction and the decarboxylation of oxaloacetate to CO_2 (32). This can also occur in L-ornithine fermentation by *B. ketoglutamicum*.

From the stoichiometric analysis, it is suggested that an enhanced L-ornithine production could be achieved by developing a new mutant which possesses a higher growth yield than the strain used in this study. In addition, a further increase in L-ornithine production can be obtained by the addition of glycine. More efforts will be made to elucidate the detailed effect of glycine on L-ornithine biosynthesis.

NOMENCLATURE

C : conversion ratio of total glycine to biomass fraction
ΔP_1* : theoretical ornithine concentration from glucose
ΔP_2* : theoretical ornithine concentration from glycine
ΔP^{total} : theoretical ornithine concentration from glucose and glycine
ΔS_1^{total} : total glucose concentration consumed
ΔS_1^{cell} : glucose concentration consumed for cell growth
ΔS_1^{omit} : glucose concentration consumed for ornithine biosynthesis
ΔS_2^{total} : total glycine concentration consumed
ΔS_2^{cell} : glycine concentration consumed for cell growth
ΔS_2^{omit} : glycine concentration consumed for ornithine biosynthesis
Y_{x1}* : intrinsic cellular yield
Y_{p1}* : theoretical ornithine yield from glucose
Y_{p2}* : theoretical ornithine yield from glycine
ΔX : cell mass

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(Received January 21, 1994)