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Effects of Medium Components on L-Ornithine Production by *Brevibacterium ketoglutamicum*

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Effects of yeast extract and ammonium sulfate were investigated on the production of L-ornithine by an arginine auxotroph, *Brevibacterium ketoglutamicum* in flask and batch cultures. Yeast extract as an arginine source and ammonium sulfate as an inorganic nitrogen source had significant effects on L-ornithine production and cell growth. L-ornithine production was repressed by the excessive addition of arginine. Reversion of auxotrophic cells to the wild type was observed when the initial yeast extract concentration was too low. There existed optimum concentrations of yeast extract and ammonium sulfate for L-ornithine production. The effects of yeast extract and ammonium sulfate concentrations on the Leudeking-Piret model parameters were examined to analyze the relationship between cell growth and L-ornithine production.

Key words: L-ornithine, *Brevibacterium ketoglutamicum*, auxotroph, L-arginine

INTRODUCTION

L-ornithine belongs to the glutamic acid family and is an intermediate metabolite in arginine biosynthesis. It is known to be effective for the treatment of liver diseases. Kinoshita *et al.* [1] reported that a citrulline-requiring mutant of *Corynebacterium glutamicum* accumulated L-ornithine with a high yield under appropriate fermentation conditions.

L-ornithine, as well as citrulline and arginine, is synthesized via glutamic acid consuming energy (ATP), hydrogen donors, and other amino acids. Therefore, L-ornithine production requires large amounts of carbon and nitrogen sources as well as aerobic culture conditions. Since L-ornithine ($C_5H_{12}O_2N_2$) contains two nitrogen atoms, the shortage of nitrogen source has an undesirable effect on its production. The shortage of ammonium ion promotes α-ketoglutaric acid production, which results in a decrease of L-ornithine yield. Although a large amount of ammonium ion is required, high ammonium concentration is inhibitory not only to the cell growth, but also to the production of L-ornithine [2].

When an arginine-auxotrophic mutant is used, arginine or arginine-containing organic nutrients such as peptone or yeast extract must be supplied. The biosynthetic pathway of L-ornithine from glutamic acid and regulatory mechanisms involved have been studied in glutamic acid-producing bacteria [3, 4, 5]. The formation of N-acetylglutamokinase, the second enzyme involved in this pathway, has been reported to be repressed by L-arginine in *Brevibacterium flavum* [6]. In addition, its activity is inhibited by an excess amount of L-arginine. Therefore, the level of L-arginine should be carefully controlled in the fermentative production of L-ornithine [7, 8, 9].

As mentioned above, there have been a number of reports on the effects of arginine or ammonium ion on L-ornithine production. However, modelling of this process has never been attempted, which would be useful for understanding the fermentation process and the pattern of L-ornithine production. In this study, the effects of yeast extract, arginine, and ammonium sulfate on L-ornithine production by *Brevibacterium ketoglutamicum* were evaluated in flask and batch cultures. Some modelling considerations were made on the kinetic relationship between ornithine production and cell growth, and on how the kinetic parameters were affected by the varying concentrations of the key components.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *Brevibacterium ketoglutamicum* 1047, which is an L-arginine or, more precisely, L-citrulline auxotrophic mutant. Stock culture was prepared in a glycerol solution (15%) and stored at -4°C.

Culture Condition

The growth medium used for inoculum was a YPD medium (glucose 2%, yeast extract 1%, peptone 1%, pH 7.0). The seed culture was prepared by growing cells in a 250 mL shake-flask containing 100 mL of medium for 12 hours. A basal medium (Table 1(A)) was used for

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shake-flask experiments. Cells were cultivated for 70 hours in a 250 mL flask containing 100 mL of medium. The initial pH of the medium was adjusted to 7.0 with an 1 N NaOH solution. During the culture, $CaCO_3$ powder was used to maintain the pH. Temperature and agitation speed were controlled at 30°C and 150 rpm, respectively. Bioreactor experiments using another basal medium (Table 1(B)) were carried out in a 2.5 L jar fermentor with a culture volume of 1.2 L. Temperature and pH were maintained at 30°C and 7.0 with NH_4OH, respectively. The addition of NH_4OH had two purposes; to adjust the pH and to supply nitrogen. Air was supplied at an aeration rate of 1 vvm and the agitation speed was kept at 600 rpm.

Analytical Methods

Cell growth was monitored by measuring optical density at 600 nm using a spectrophotometer (MODEL 930, UVICON, USA). In flask cultures, remaining $CaCO_3$ powder was dissolved by adding a 0.1 N HCl solution to avoid interference in measuring optical density for dry cell weight estimation. Glucose concentration was measured using an enzyme kit (Gluco-E Kit, Young-Dong Pharmaceutical. Co., Seoul, Korea). L-ornithine concentration was determined by the method described by Chinard [10].

RESULTS AND DISCUSSION

Flask Culture

Effects of several key medium components on the cell growth and L-ornithine production were investigated. They included yeast extract, ammonium sulfate, L-arginine, KH_2PO_4, and $NaHPO_4$. Glucose was the sole carbon source, and ammonium sulfate and yeast extract were the nitrogen sources. Yeast extract was also the source of arginine and other organic nutrients.

Table 1. Compositions of basal media

Ingredient	(A)	(B)
glucose	50	100
yeast extract	10	10
$(NH_4)_2SO_4$	10	20
KH_2PO_4	0.75	0.75
Na_2HPO_4	1.5	1.5
$MgSO_4 \cdot 7H_2O$	1.0	1.0
$MnSO_4 \cdot 4H_2O$	0.05	0.05
$FeSO_4 \cdot 7H_2O$	0.01	0.01
$ZnSO_4 \cdot 7H_2O$	0.01	0.01
$CaCO_3$	5	
pH	7.0	7.0

(A) production medium for flask culture
(B) production medium for batch culture in bioreactor

The effect of yeast extract concentration

The initial concentrations of yeast extract tested were 0, 5, 10, 20, and 30 g/L. The final cell concentration increased almost linearly with increasing yeast extract concentration, and was 15.5 g/L for 30 g/L of yeast extract. As shown in Table 2, the maximum final L-ornithine concentration obtained was 8.8 g/L when the initial concentration of yeast extract was 5 g/L The product yield based on glucose ($Y_{p/s}$) was 0.220. L-ornithine production decreased with the yeast extract concentration when higher than 5 g/L because an excessive amount of arginine in the yeast extract inhibited and repressed the enzymes involved in L-ornithine biosynthesis. The ratio of product to cell mass formation ($Y_{p/x}$) was highest when the initial concentration of yeast extract was 5 g/L (Table 2). On the contrary, the cellular yield showed a minimum at this concentration of yeast extract.

The effect of ammonium sulfate concentration

The initial concentrations of ammonium sulfate tested were 0, 10, 20, 30 and 50 g/L. Cell growth was negligible without the addition of ammonium sulfate (Table 2). When excessive ammonium sulfate was added, cell growth was slightly inhibited. The maximum L-ornithine concentration was 7.9 g/L when the initial ammonium sulfate concentration was 10 g/L. On the other hand, the maximum cell concentration was 6.9 g/L at an ammonium sulfate concentration of 20 g/L. The maximum cellular and product yields were 0.197 (for ammonium sulfate 20 g/L) and 0.169 (for ammonium sulfate 10 g/L), respectively. The highest $Y_{p/x}$ of 1.20 was obtained for 10 g/L of ammonium sulfate. We can see from Table 2, that the production of L-ornithine was more significantly affected by the ammonium sulfate concentration than cell growth.

The effect of L-arginine addition

Arginine, although necessary for cell growth, inhibited and repressed the enzymes involved in the production of L-ornithine, as explained above. Therefore, it was important to find the optimum initial concentration of arginine. The concentrations of arginine added were 0, 0.1, 0.3, 0.5, 1.0, and 2.0 g/L (Table 3). Cell concentration increased with the addition of ar-

Table 2. Effects of yeast extract and ammonium sulfate in flask culture

		Dry cell weight (g/L)	Ornithine (g/L)	Residual glucose (g/L)	$Y_{p/s}$ (g-ornithine/ g-glucose consumed)	$Y_{x/s}$ (g-cell/ g-glucose consumed)	$Y_{p/x}$ (g-ornithine/ g-cell)
Yeast extract (g/L)	0.0	0.3	0.0	48.0	0.000	0.220	0.00
	5.0	3.5	8.8	12.2	0.220	0.086	2.50
	10.0	6.2	3.4	15.7	0.095	0.172	0.55
	20.0	10.8	0.4	18.4	0.013	0.320	0.04
	30.0	15.5	0.0	20.5	0.000	0.490	0.00
$(NH_4)_2SO_4$ (g/L)	0.0	0.6	0.0	28.8	0.000	0.028	0.00
	10.0	6.6	7.9	3.3	0.169	0.141	1.20
	20.0	6.9	4.2	14.9	0.120	0.197	0.61
	30.0	6.6	2.9	14.1	0.082	0.184	0.44
	50.0	6.2	0.7	13.8	0.021	0.171	0.11

ginine, but the increase was not proportional. Some of the other nutrients than arginine seemed to be insufficient for more cell growth when the arginine concentration was higher than 1.0 g/L. The maximum final L-ornithine concentration of 7.9 g/L was obtained for an initial arginine concentration of 0.3 g/L. As in the case of yeast extract, L-ornithine production was severely affected by arginine concentration. The reason why an excessive addition of yeast extract decreased the production in previous experiments could be explained by the effect of L-arginine in yeast extract.

The effect of KH_2PO_4

The concentrations of KH_2PO_4 tested were 0, 0.5, 1.0, 1.5, and 2.0 g/L. Similar levels of L-ornithine production (7.8-8.9 g/L) were obtained in the range of 0-1.5 g/L of KH_2PO_4 (Table 3). Also, there was no significant variation in cell growth (*ca.* 6.2 g/L) when the initial concentrations of KH_2PO_4 were in this range. However, excessive addition of KH_2PO_4 (2.0 g/L) was inhibitory to the cell growth and L-ornithine production. Cells grew well and produced L-ornithine to a significant amount without the addition of KH_2PO_4. This seemed to be due to the phosphate and potassium ions contained in yeast extract.

The effect of Na_2HPO_4 concentration

The concentrations of Na_2HPO_4, tested were 0, 0.5, 1.0, 1.5, and 2.0 g/L. The maximum L-ornithine concentration of 9.4 g/L was obtained when the initial concentration of Na_2HPO_4 was 1.5 g/L (Table 3). Similar levels of L-ornithine production were obtained in the range of 1.0-2.0 g/L of KH_2PO_4. There was no significant variation in cell growth (ca. 5.2 g/L) when the initial concentrations of KH_2PO_4 were in the range of 1-1.5 g/L. But in the case of excessive addition (over 1.5 g/L), cell growth was inhibited. Cells grew and produced L-ornithine to a significant amount without the addition of Na_2HPO_4.

Batch Culture in a Bioreactor

Table 3. Effects of arginine, Na_2HPO_4, and KH_2PO_4 in flask culture

		Dry cell weight (g/L)	Ornithine-HCl (g/L)
L-arginine (g/L)	0.0	0.1	0.0
	0.1	4.5	4.8
	0.3	4.9	7.9
	0.5	6.3	6.7
	1.0	7.0	3.0
	2.0	7.1	0.5
KH_2PO_4 (g/L)	0.0	6.2	7.7
	0.5	5.6	9.2
	1.0	6.6	9.0
	1.5	6.5	7.7
	2.0	4.8	4.7
Na_2HPO_4 (g/L)	0.0	4.9	6.1
	0.5	4.7	7.2
	1.0	5.7	9.0
	1.5	4.6	9.4
	2.0	4.3	8.9

*If arginine contained in 1g of yeast extract is assumed to be 0.03 g, the yeast extract concentrations equivalent to these L-arginine concentrations are 0.0, 3.3, 10.0, 16.6, 33.3, and 66.7 g/L

We investigated in detail how glucose consumption, cell growth, and L-ornithine production changed with varying concentrations of yeast extract and ammonium sulfate. As in flask cultures, critical concentrations were clearly found for both yeast extract and ammonium sulfate.

The effect of yeast extract

The initial concentrations of yeast extract tested were 20, 10, and 5 g/L (Fig. 1). Cells grew well with 20 g/L of yeast extract (Fig. 1(A)). The final cell concentration was as high as 31 g/L, but the concentration of L-ornithine was only 17.5 g/L because the excessive arginine from yeast extract inhibited the biosynthesis of L-ornithine. At the beginning of the culture, L-ornithine was not produced due to the high concentra-

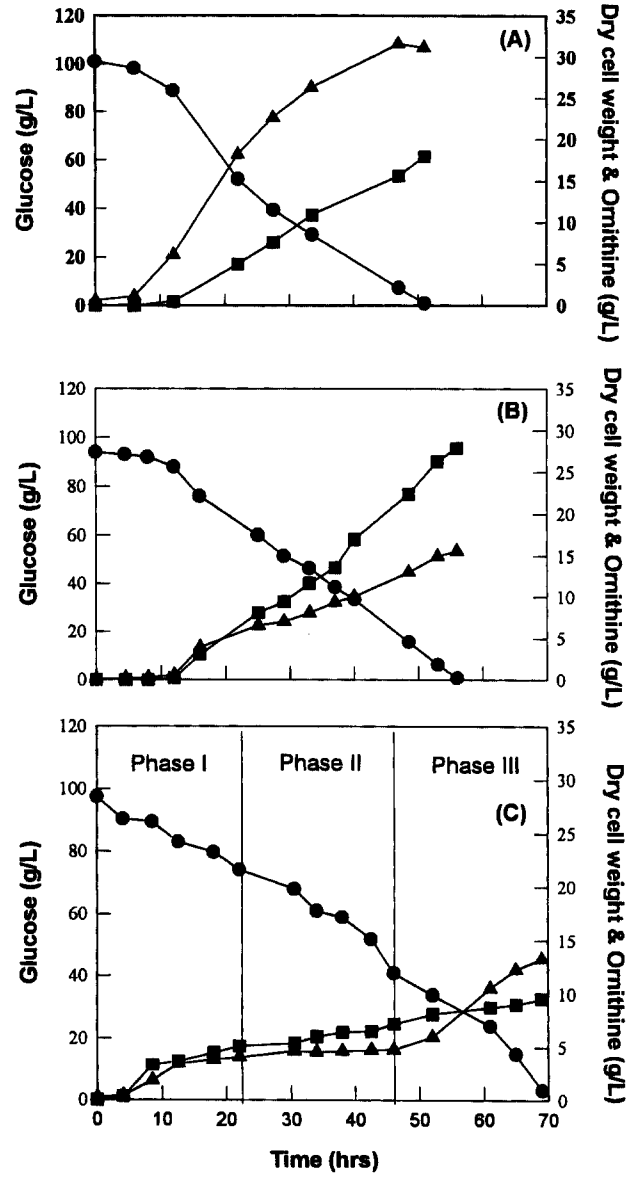

Fig. 1. Time courses of ornithine production and cell growth for various yeast extract concentrations in batch cultures in bioreactor.(yeast extract effect)
(A) yeast extract 20 g/L, ammonium sulfate 20 g/L, (B) yeast extract 10 g/L, ammonium sulfate 20 g/, (C) yeast extract 5 g/L, ammonium sulfate 20 g/L, ■ : ornithine ▲: dry cell weight ● : glucose

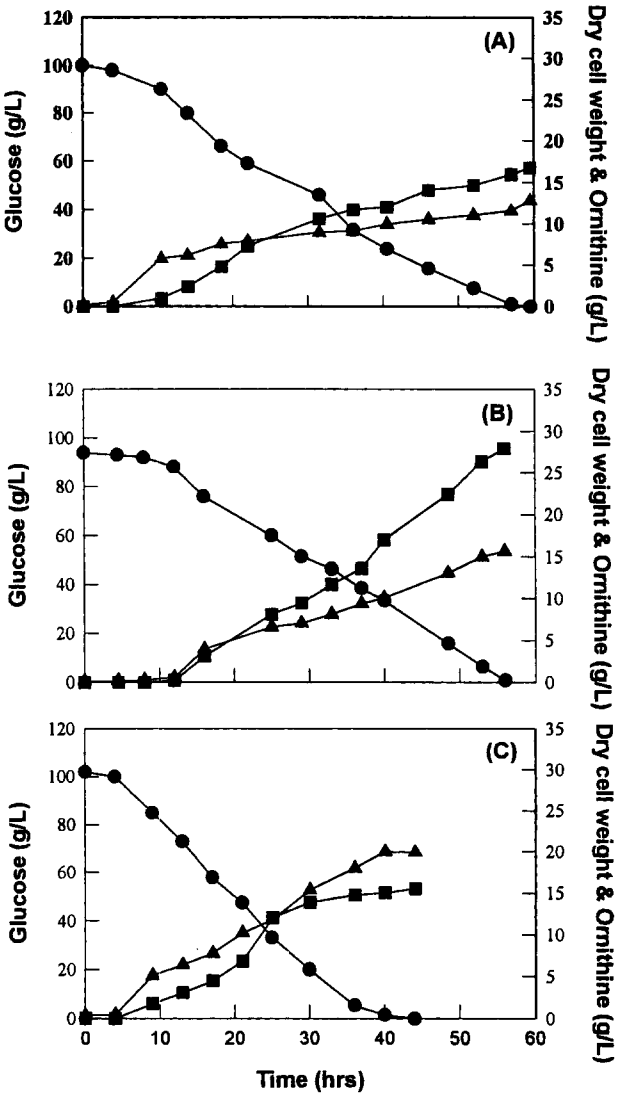

Fig. 2. Time courses of ornithine production and cell growth for various ammonium sulfate concentrations in batch cultures in bioreactor (ammonium sulfate effect).
(A) ammonium sulfate 30 g/L, yeast extract 10 g/L, (B) ammonium sulfate 20 g/, yeast extract 10 g/L, (C) ammonium sulfate 10 g/L, yeast extract 10 g/L, ■ : ornithine ▲: dry cell weight ● : glucose

tion of arginine. After 12 hours, L-ornithine started to be produced. Glucose (100 g/L) was completely consumed in 52 hours.

As shown in Fig. 2(B), cultivation with a lower concentration of yeast extract (10 g/L) resulted in a higher concentration of L-ornithine(28.3 g/L). The final cell concentration was 17 g/L, which was much lower than that obtained with 20 g/L of yeast extract. Glucose was completely consumed in 55 hours.

Fig. 1(C) shows the results for 5 g/L of yeast extract. This concentration was found to be the optimum in the flask culture in which a lower glucose concentration of 50 g/L was used. Cell concentration increased exponentially to 4 g/L (Phase I), and thereafter cell growth was retarded for 30 hours due to the depletion of yeast extract (Phase II). Cell concentration again increased after 46 hours (Phase III). This was due to the appearance of revertant cells that lost the characteristics of an auxotroph. A low production of L-ornithine (10 g/L) was obtained due to the growth of revertant cells and the inhibition of arginine produced by the revertant cells.

The effect of varying concentration of yeast extract is summarized in Table 4. The mean cell concentration ($\bar{X}$) is defined as the average of the initial and final cell concentrations in each run or each phase of a run, which is roughly the half of the final concentration since the initial cell concentration is negligible. Such definition of $\bar{X}$ can be justified since the profiles of the cell concentration are almost linear. Here, a systematic analysis is in need, especially for the case of 5 g/L of yeast extract concentration, which showed a rather complex nature. Ornithine is a primary metabolite and its production is closely associated with cell growth. If we assume ornithine production is solely dependent on cell growth, the rate of ornithine production can be represented by

$$\frac{dP}{dt} = \frac{dX}{dt} \tag{1}$$

where α is a proportional constant. This equation can be recast in an integrated form, $\triangle P \simeq \alpha \triangle X$. From this we know that $Y_{P/X} = \frac{\triangle P}{\triangle X} \simeq \alpha$. Therefore, if the above assumption is true, the values of YP/X for Phase I and Phase II should be, even though roughly, the same. But, the value for Phase II is considerably higher than that for Phase I. This suggests an augmented expression is in need for the analysis of ornithine production characteristics. For this reason, we suggest the Leudeking-Piret kinetics which is represented by

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{2}$$

This can be recast in an integrated form

$$\triangle P = \alpha \triangle X + \beta \bar{X} \triangle t \text{ or } Y_{P/X} = \alpha + \beta \frac{\bar{X} \triangle t}{\triangle X} \tag{3}$$

Following this expression, the higher value YP/X for Phase II is due to the larger value of the second term, $\beta \frac{\bar{X} \cdot \triangle t}{\triangle X}$. This is thought to be due to both an increased value of β and an increased value of $\frac{\bar{X} \cdot \triangle t}{\triangle X}$ (11.0 for Phase I, 118.7 for Phase II). However, the first part of the argument that an increased β causes the increased $Y_{P/X}$ is not clear yet. It can be also explained by analyzing the data for the cases of 10 and 20 g/L of yeast extract concentrations. For both cases, the term $\beta \frac{\bar{X} \cdot \triangle t}{\triangle X}$ has the similar value. But, $Y_{P/X}$ for the case of 10 g/L is much higher than that for 20 g/L. This is solely due to the larger value of β for the case of 10 g/L. The reason is, as mentioned earlier, that the concentration of arginine contained in yeast extract maintained at a lower level than the case of 20 g/L of yeast extract concentration. In conclusion, ornithine formation can be represented by a Leudeking-Piret type kinetic expression in which the parameter β is negatively dependent on the arginine concentration. The low value of $Y_{P/X}$ for Phase III seems to be due to the appearance and active growth of nonproductive revertant cells and thus a totally different analysis should be done for this.

The effect of ammonium sulfate

The initial concentrations of ammonium sulfate

Table 4. Summary of bioreactor experiments

			△t (hrs)	Dry cell weight formed △X (g/L)	Average cell concentration $\bar{X}$ (g/L)	Ornithine produced △P (g/L)	$Y_{p/s}$ (g-ornithine/ g-glucose consumed)	$Y_{x/s}$ (g-cell/ g-glucose consumed)	$Y_{p/x}$ (g-ornithine/ g-cell)	$\frac{\bar{X}\cdot\triangle t}{\triangle X}$	$\frac{\triangle P}{\bar{X}\cdot\triangle t}$	$\frac{\triangle X}{\bar{X}\cdot\triangle t}$
Yeast extract (g/L)	5.0	Phase I	22	4.1	2.05	5.1	0.252	0.203	1.24	11.0	0.113	0.091
		Phase II	24	0.7	4.45	1.4	0.050	0.025	2.00	152.6	0.013	0.007
		Phase III	24	8.5	9.05	3.6	0.069	0.163	0.42	25.6	0.016	0.039
		Overall	70	13.3	6.65	10.1	0.101	0.133	0.76	35.0	0.023	0.029
	10.0		56	17.0	8.50	28.3	0.283	0.170	1.66	28.0	0.059	0.036
	20.0		51	31.0	15.5	17.5	0.175	0.310	0.56	25.5	0.022	0.039
$(NH_4)_2SO_4$ (g/L)	10.0		45	20.2	10.10	15.0	0.150	0.202	0.74	22.5	0.033	0.044
	20.0		56	17.0	8.50	28.3	0.283	0.170	1.66	28.0	0.059	0.036
	30.0		60	13.1	6.55	17.0	0.170	0.131	1.30	30.0	0.043	0.033

were 30, 20, and 10 g/L. When the initial ammonium sulfate concentration was 30 g/L, the final concentrations of cell and L-ornithine were 13.0 and 17.0 g/L, respectively (Fig. 2(A)). Glucose was depleted in 59 hours. L-ornithine production was enhanced for 20 g/L of ammonium sulfate, but and cell concentration decreased as seen in Fig. 2(B). The final L-ornithine and the cell concentrations were 28.3 and 17.0 g/L, respectively. Glucose was depleted in 55 hours.

In the case of 10 g/L of ammonium sulfate concentration (Fig. 2(C)), there was no retardation of cell growth. The final L-ornithine concentration was 15.0 g/L and the cell concentration was 20.2 g/L. The L-ornithine concentration rapidly increased until 25 hours. Thereafter, the rate of increase was smaller than that of the initial period due to the depletion of nitrogen sources. Glucose was completely consumed in 43 hours.

The effect of varying concentration of ammonium sulfate are also summarized in Table 4. We can see from the △X data that cell growth decreased with increasing concentration of ammonium sulfate. For the purpose of evaluating the effect on the specific production rate, β, the Leudeking-Piret type expression can be rewritten as $\frac{\triangle P}{\bar{X}\cdot\triangle t}=\alpha\frac{\triangle X}{\bar{X}\cdot\triangle t}+\beta$. The term, $\frac{\triangle P}{\bar{X}\cdot\triangle t}$ shows a maximum for 20 g/L of ammonium sulfate concentration. If we consider the term, $\frac{\triangle X}{\bar{X}\cdot\triangle t}$ slightly decreases with the ammonium sulfate concentration, it is clear that β has a maximum. In other words, there is an optimum ammonium sulfate concentration for the specific production rate.

Nomenclature

P : L-ornithine concentration (g/L)
X : Cell concentration (g/L)
△t : Time period for each culture or phase (hr)
△P : L-ornithine formed (g/L)
△X : Change of cell concentration (g/L)
$\bar{X}$: Mean cell concentration (g/L)
$Y_{X/S}$: Yield of cell from substrate, $\frac{\triangle X}{\triangle S}$
$Y_{P/S}$: Yield of L-ornithine from substrate, $\frac{\triangle P}{\triangle S}$
$Y_{P/X}$: Ratio of product to cell mass, $\frac{\triangle P}{\triangle X}$
α,β : Coefficients in Leudeking-Piret model

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