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Optimization of an Intact Cell System of *Rhodocyclus gelatinosus* KUP-74 for δ-Aminolevulinic Acid Production

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A novel system has been developed to produce δ-aminolevulinic acid (ALA) using the intact cells of late logarithmic phase of *Rhodocyclus gelatinosus* KUP-74. The system was shown optimum yield of extracellular ALA under a condition of anaerobic light irradiation (4 Klux) at 30℃ with no variation in cell mass. The rate of extracellular ALA formation was stimulated by low doses of either C_4 or C_5 ALA biosynthetic precursors, where 5 mM (C_4) and 3 mM (C_5) of each precursors were appeared to generate the maximum rates of 3.3 and 4.0 nmoles of ALA per 0.35 mg cells per hr, respectively. Half-life of the system was 10 hr in a sense of an ability of portage transport of L-glutamate, and sequential dose of this compound was resulted in promising recovery of the ALA.

In recent studies, it has been proved that δ-aminolevulinic acid (ALA), a prime intermediate in the cyclic tetrapyrrole biosynthesis (25), functions effectively as a lethal mean for a number of targets, e.g., skin cancers and weeds (20, 23), because it becomes a form of pchlide under an appropriate extra source of light. Although a considerable amount of effort has been directed toward mass production of the compound from this point of view, the provision of ALA from the sources of supply are far from the demands yet (24).

ALA can be generated either by C_4 or C_5 ALA biosynthetic pathways in living cells (9). By the former pathway, found in animal (14, 18) and some bacterial cells (16, 22, 28), glycine and succinyl-CoA are condensed directly to ALA with a liberation of CO_2 in the presence of ALA synthase, a pyridoxal phosphate-dependent enzyme. In the latter one, found mainly in plant (4, 5), algal (1, 12), and some archaebacterial cells (3, 11), the intact carbon skeleton of L-glutamate is placed to ALA through a sequence of reaction by three enzymes along with tRNA-Glu, ATP and $NADPH+H^+$.

Since the ALA biosynthetic step is a major committed point, the intracellular concentration of ALA is known to be strictly restricted by certain factors such as ALA dehydratase or end products (27). From this reason, it has been thought to be fairly difficult to yield out *in vivo* for general use. Therefore, current concern with respect to ALA is focused continuously on the development of novel procedures for its efficient production.

For this purpose, we examined to investigate whether a rational system using the intact cells of *Rhodocyclus gelatinosus* provides a selective generation of ALA. In this paper, we will also describe the possible directions of mass production of ALA by this methodology.

MATERIALS AND METHODS

Microorganism and Chemicals

A photosynthetic bacterium, *Rhodocyclus gelatinosus* KUP-74 (13), isolated from a soil sample of Chung-Nam prefecture (Korea), was used for the intact cell reaction. Unless otherwise indicated, all chemicals were commercial preparations of analytical reagent grade. δ-Aminolevulinic acid (ALA), levulinic acid (LA), *p*-dimethylaminobenzaldehyde, 2, 4, 6-trinitrobenzenesulfonate (TNBS), and L-glutamate were purchased from Sigma Chem. Co. (U.S.A). Glycine, succinic acid, and 70% perchloric acid were obtained from Junsei Chem. Co. (Japan). Glacial acetic acid was obtained from Duksan Pharm. Co. (Korea).

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Medium and Culture Conditions

Strain KUP-74 was grown on a glass water bath anaerobically at 30℃ under 4 Klux of light irradiation in a culture tube (2×20 cm) containing 10 m*l* of Lascelles' basal medium (15) consisted of 2.7 g of D,L-malic acid, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.8 g of $(NH_4)_2HPO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 27 mg of $CaCl_2 \cdot 2H_2O$, 1 mg of nicotinic acid, 1 mg of thiamine-HCl, 1.2 mg of $MnSO_4 \cdot 5H_2O$ and 0.01 mg of biotin in 1 *l* of distilled water with a pH of 6.8 adjusted by conc. NaOH.

Preparation of Intact Cells

Strain KUP-74 was grown in the basal medium described above to the late logarithmic phase (1×10^9 cells per m*l*). It was then harvested by centrifugation (10,000 rpm for 10 min), and washed twice by resuspension in 10 mM phosphate buffer (pH 7.0) and centrifuged. The cells were then resuspended in the same buffer to a concentration of 0.35 mg dry weight cells per m*l* ($A_{660}=1.0$). Concentration of dry weight of cells per m*l* was determined by measuring the absorbance at 660 nm, and was compared to a standard curve. The cell suspension prepared was used directly without any prolonged storage.

Intact Cell Reaction

The standard reaction mixture consisted of 5 mM LA, 0.35 mg dry weight cells, and 10 mM phosphate buffer (pH 7.0), in a final volume of 1 m*l*. The reaction was followed at 30℃ anaerobically under the condition of 4 Klux of light source. The rate of extracellular ALA formation was determined by a progressive 1 hr time-interval assay and was expressed as nmoles per m*l* per hr.

ALA Analysis

ALA analysis was performed according to the method of Mauzerall, *et al.* (19) as follows: Portions of the cell suspension were centrifuged immediately or at fixed time-intervals, and 0.5 m*l* of each of the supernatants were then transferred to screw-cap tubes. To this, 0.5 m*l* of 1 M Na-acetate buffer (pH 4.7), 0.05 m*l* of acetylacetone were successively added, followed by heating in a boiling water bath for 15 min to form 2-methyl-3-acetyl-5-propionic acid pyrrole. Colored adduct formed in the presence of 3.5 m*l* of modified Ehrlich reagent (26) containing 1 g of *p*-dimethylaminobenzaldehyde in 42 m*l* of glacial acetic acid and 8 m*l* of 70% perchloric acid was shown a maximum absorbance at 556 nm, and the absorbance was converted to nmoles per m*l* by using the molar extinction coefficient for the colored adduct of 9.3×10^3 $M^{-1}cm^{-1}$ (13).

Determination of Cellular Uptake Rate of L-Glutamate

Intact cells obtained by the above procedure were homogeneously suspended to be 0.35 mg in 10 mM phosphate buffer (pH 7.0), and were placed in culture tubes (1×10 cm) and 1 mole of L-glutamate was added as appropriate to give a final volume of 1 m*l*. Portions of the suspensions (100 μ*l*) were centrifuged immediately or at 2 min time-intervals in the course of incubation at 30℃ under 4 Klux of light source. The residual amounts of L-glutamate were determined from the fractional supernatants by following procedure: 100 μ*l* of each of the aqueous phases and 10 μ*l* of 40 mg/m*l* TNBS were added to 5% sodium borate solution (pH 9.5), to give final volumes of 1 m*l* each. After incubation at 30℃ for 30 min, the amount of picryl adducts formed were determined by measuring the absorbance at 420 nm ($\varepsilon_{420}=1.0\times10^4$ $M^{-1}cm^{-1}$) (21).

Miscellaneous Assays

Protein content was determined by using the method described by Lowry, *et al* (17). Sugar content was assayed by a modified method of Whistler and Bemiller (29). Nucleic acid and pigment contents were determined spectrophotometrically by using a Hitachi model spectrophotometer 100−30. The amount of primary amino group was estimated by using the TNBS method (21) described above.

RESULTS

Preparation of Intact Cells for Extracellular ALA Formation

Alteration in cell mass should be undesirable for selective isolation of the ALA because of a possible incidence of increasing impurities. An experiment was carried out to determine whether the intact cells do vary in their mass during ALA secretion. The result shown in Fig. 1 reveals that no apparent cell growth was observed except in the case of the cells from mid logarithmic phase. And also, it was assumed that the cell growth in this system may be counteracted in ALA production. The effect of cell concentration on the rate of extracellular ALA formation was investigated by using the cells of late logarithmic phase. Fig. 2 shows a constant increase of the rate of extracellular ALA formation in proportion to cell concentration, suggesting that the amount of extracellular ALA could be determined reliably at least within the range of cell concentration employed. For convenience, 0.35 mg cells per m*l* ($A_{660}=1.0$) were used for initiating the reactions.

Extracellular accumulation of primary amines, especia-

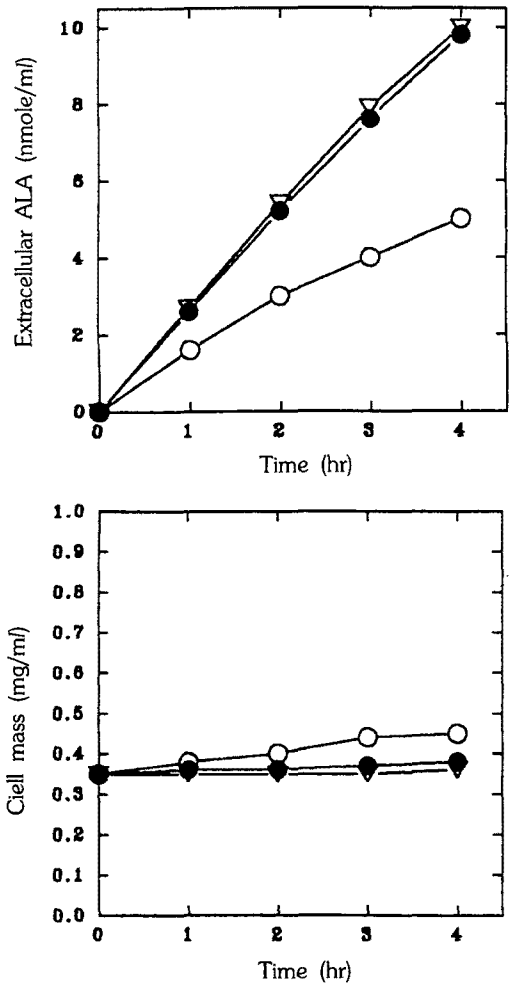

Figure 1. Changes in cell mass and extracellular ALA amount during the intact cell reactions.
Cells of mid logarithmic (○), late logarithmic (●) and stationary (▽) phases were used as intact cells. The reaction was carried out under the standard condition as described in Materials and Methods.

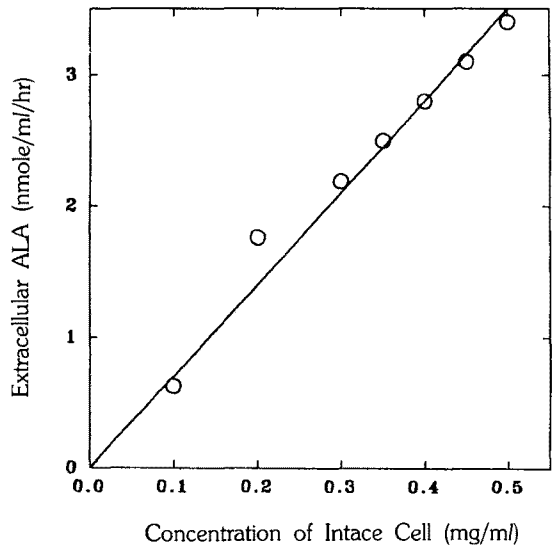

Figure 2. Relationship between concentration of the intact cells and the rate of extracellular ALA formation.
Cells of late logarithmic phase were used as intact cells. The reaction was followed under the standard condition using fixed concentrations of intact cells (0.1~0.5 mg) per ml and the rate of extracellular ALA formation was determined by progressive 1 hr time-interval assay method

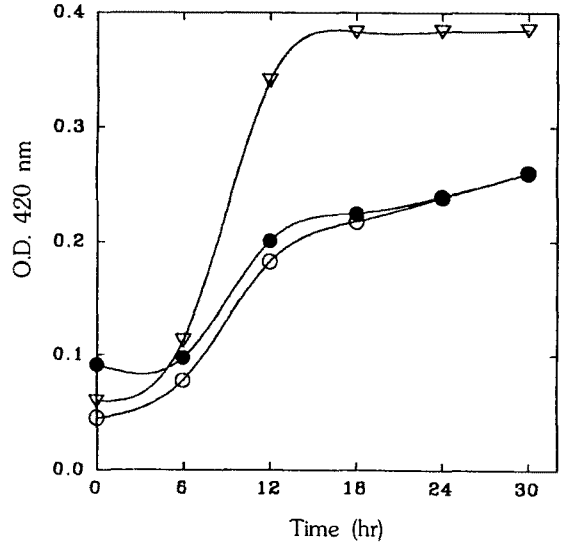

Figure 3. Release of compounds containing primary amino groups during incubation of the intact cells.
Cells of mid logarithmic (○), late logarithmic (●), stationary (▽) phases were used as intact cells. The extracellular amount of primary amines were determined by measuring the optical density (A_{420}) of picryl adducts, formed in the presence of TNBS, as described in Materials and Methods.

lly amino acids, other than ALA itself should be avoided if possible. Fig. 3 illustrates the differential increases of extracellular contents of these compounds from the various intact cells. An exponential accumulation of these compounds was found in the intact cells of stationary phase. Further experiments were carried out to compare the extracellular contents of undesirable organic matters, and the results are summarized in Table 1. Among the intact cells examined, maximum amount of ALA was obtained by the intact cells of late logarithmic phase along with similar distributions of other compounds as that found in the intact cells of other growth phase. This system was conceivably far more efficient in generating extracellular ALA than the cultivation method in that the former method only required 3 mM L-glutamate to give the maximum yield of extracellular ALA whereas

Table 1. Extracellular production of organic matters from intact cell system

	Intact cell system[a] (Growth phase)			Cultivation[b]
	Mid-log	Late-log	Stationary	
ALA (μM)	11.5	25	15.2	117
A_{280} nm	0.571	0.483	0.535	1.310
A_{260} nm	0.412	0.390	0.490	1.535
Proteins (μg/ml)	70	54	60	182
Carbohydrates (μg/ml)	48	42	39	220
Lipids (mg/ml)	0.3	0.6	0.6	1.7
Primary amine	5.70	7.93	5.42	30.5
Pigments (530 nm)	0.042	0.040	0.047	0.071

[a] Each preparations of intact cells at various growth phase were incubated for 20 hr under the standard condition described in Materials and Methods except the reaction systems were composed by 5 mM LA and 3 mM L-glutamate in 10 mM phosphate buffer, pH 7.0. [b] Culture broth grown anaerobically at 30℃, 4 klux for 5 days on Lascelles' basal medium (pH 7.0) containing 10 mM LA and 30 mM L-glutamate was centrifuged and the resulted supernatant was directly used for above analysis.

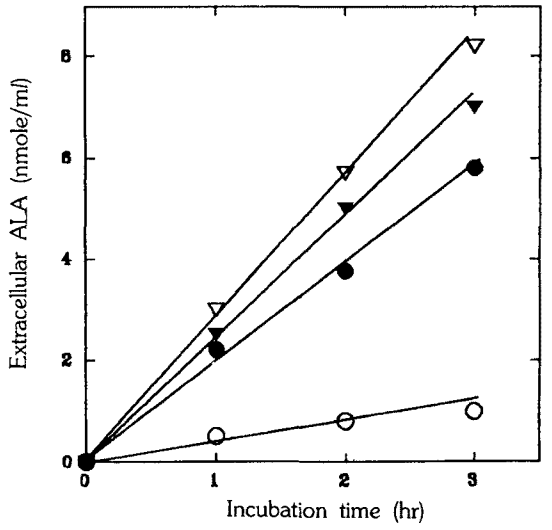

Figure 4. Effect of light intensity on extracellular ALA formation during the intact cell reactions.
The reactions were carried out at 30℃ under light irradiation of 0 (○), 2 (●), 4 (▽) and 6 (▼) Klux.

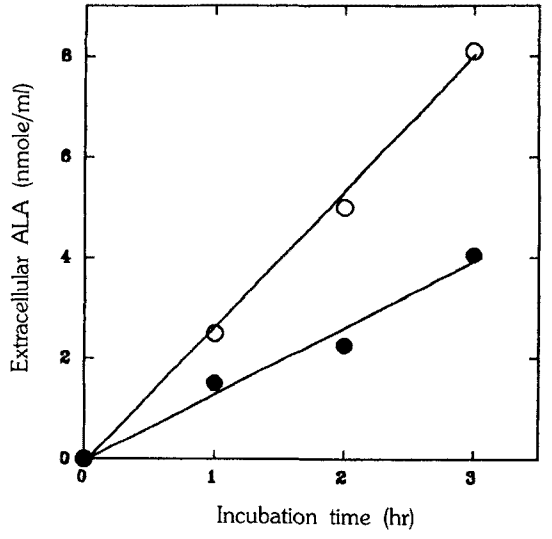

Figure 5. Extracellular ALA formation in intact cell system under anaerobic and aerobic conditions.
The reactions were performed at 30℃, 4 Klux under anaerobic (○) or aerobic (●) conditions. To maintain anaerobic state of the system, the culture tube was sealed after extensive replacement of air phase by nitrogen gas *in vacuuo*.

the latter one needed 10 times of L-glutamate concentration than that of the former method to observe it. In consequence of the results observed above, we selected the intact cells of late logarithmic phase culture for developing a rational system of ALA production.

Physical Factors Influencing Extracellular ALA Formation

In general, the appearance of chlorophylls in the photosynthetic bacteria is strongly inhibited under the extra source of light. And, when the TCA cycle functions, then the concentration of oxygen molecule will become as a limiting factor on the accumulation of pigments, especially chlorophylls. The effect of light intensity and concentration of oxygen molecule on extracellular ALA formation of the intact cell system is illustrated in Fig. 4 and Fig. 5, respectively. As indicated, it was suggested that the light irradiation should be an essential factor to accelerate ALA production. Over 6-fold of ALA was induced under 4 Klux of light irradiation. On the other hand, the accumulation of extracellular ALA was reduced approximately 50% in the presence of atmospheric concentration of oxygen molecule. Optimal pH and temperature for the maximum yield of extracellular ALA were pH 7.0 and 30℃, respectively (data not shown).

Effect of Chemicals on Extracellular ALA Formation.

Although levulinic acid (LA) has been widely used as an essential mean in the search for a biotechnological production of ALA, the concentration in common use is unusually high (10), and the minimum concentration required to satisfy the maximum rate of ALA formation is often ignored. This problem was solved by using an intact cell preparation, which utilized less than 5 mM LA to meet maximum rate of extracellular ALA as shown in Fig. 6. Interestingly, the rate was maintained constantly while the supplemented LA concentration was increased

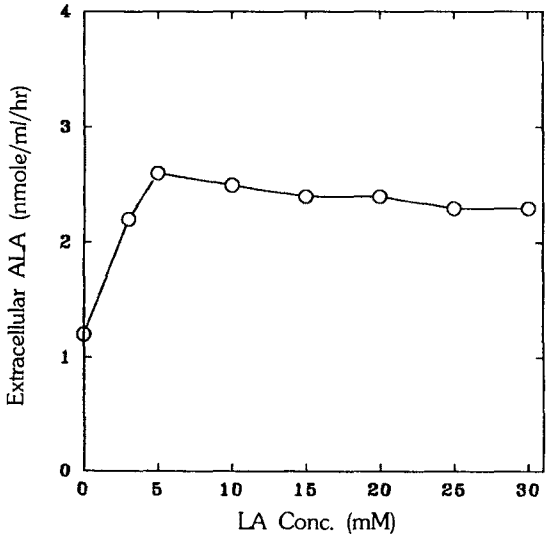

Figure 6. Effect of LA concentration on the rate of ALA formation.
The rate of extracellular ALA formation was measured using the standard assay condition described in Materials and Methods.

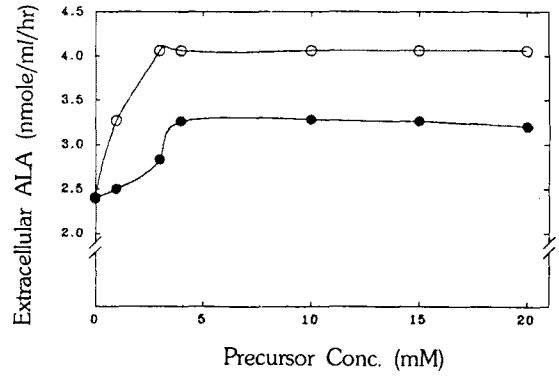

Figure 7. Effect of concentrations of ALA biosynthetic precursors on the rate of ALA formation.
L-Glutamate (○) or glycine and succinate (●) were added respectively to the standard reaction mixtures in the range of concentrations indicated in Figure. Precedure for analysis of the rates of extracellular ALA formation were described in Table 2.

Table 2. Effect of C_4 and C_5 precursors on the rate of extracellular ALA formation.

Precursors (mM)			ALA formation (nmole/ml/hr)
glycine	succinate	L-glutamate	
0	0	0	2.62
5	5	0	3.26
0	0	3	4.06
5	5	3	4.10

Intact cell reactions were carried out for 20 hr in the presence of precursors listed and 5 mM LA. The corresponding initial rates were estimated by extrapolation of progressive decreases in the rates of extracellular ALA formations determined by 1 hr time-interval.

to 5-fold or even more.

In our previous paper (7), we strongly suggested that this bacterium could synthesize ALA *in vitro* via either C_4 or C_5 ALA biosynthetic pathway. And also, it was noted that excess amounts of precursors had been used unnecessarily to observe maximum yields of extracellular ALA from the vegetative culture. Furthermore, it was not easy to determine the maximum capacity of intracellular use of each precursors within the unit time by using the above method. However, a novel information about the cellular capacity of precursor digestions could be derived from the intact cells. As shown in Fig. 7, the intact cells received only limited amounts of exogeneous source of either precursors for ALA biosynthesis, those being 3 mM, 5 mM of C_5 and C_4 precursors, respectively, to meet maximum rates of extracellular ALA formation. The relative induction ratio of extracellular ALA forming rate by C_5 precursor to that found by C_4 precursors was 1.20. And the simultaneous presence of both C_4 and C_5 precursors did not synergistically induce the rate, indicating that the expression of both pathways would be affected under mutual regulation (7) as described in the previous report (Table 2).

Effectiveness of Intact Cells

It is obviously very important to know the life span of the intact cells prior to the sequential feeding process of ALA precursors so that the desired successive production of ALA can be observed. An experiment to determine half life of the intact cells was carried out by measuring the uptake rate of C_5 precursor. As shown in Fig. 8, L-glutamate was rapidly transported until certain concentration (0.17 mM), followed by an immediate decrease of the rate. The total amount of L-glutamate driven into the cells was estimated to be 0.02 moles. The above measurement was carried out in the course of incubation of the intact cells. As shown in Fig. 9, the rate of L-glutamate uptake was gradually decreased to half rate assuming as half life of the intact cells for the precursor uptake by 10 hr of reaction. The half life of the intact cells did not necessarily indicate the cell viability since about 80 percent of the cells were still viable after 30 hr of incubation (data not shown).

The ALA yield could be enhanced by a sequential feeding of the precursor throughout the life span period for the precursor uptake. 1 mM L-glutamate was fed 3 times as indicated in Fig. 10. By this feeding system the amount of extracellular ALA was increased to 3-fold

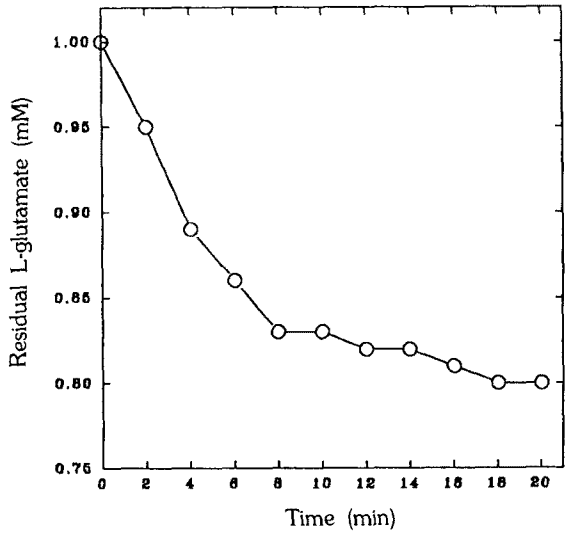

Figure 8. Determination of cellular uptake rate of L-glutamate in intact cell system.

1 mM of L-glutamate was added to the intact cell system. 100 μl of the cell suspension was centrifuged immediately, or at 2 min time. intervals, and the residual amount of L-glutamate in cell-free supernatant was determined using TNBS as described in Materials and Methods.

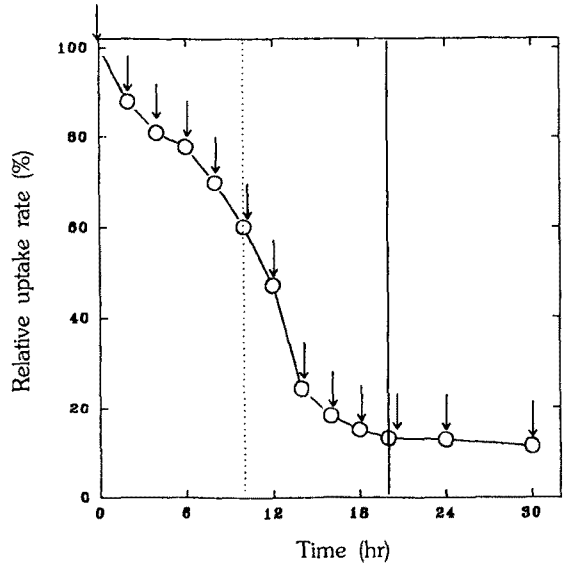

Figure 9. Life span of intact cells with the mode of cellular uptake ability for L-glutamate.

The intact cell system in the presence of 1 mM L-glutamate was incubated for 20 min as described in Fig. 8, and the transported amount of L-glutamate was analyzed. After incubation for certain period, the system was washed twice by resuspension in 10 mM phosphate buffer (pH 7.0) and centrifuged, then the same procedures were repeated at the fixed time-intervals as indicated by arrows. The capacities of L-glutamate uptakes of each intact cells examined were presented by percent rates relative to that found in fresh preparation. Dotted, solid lines indicate the points of half life and limited life span, respectively.

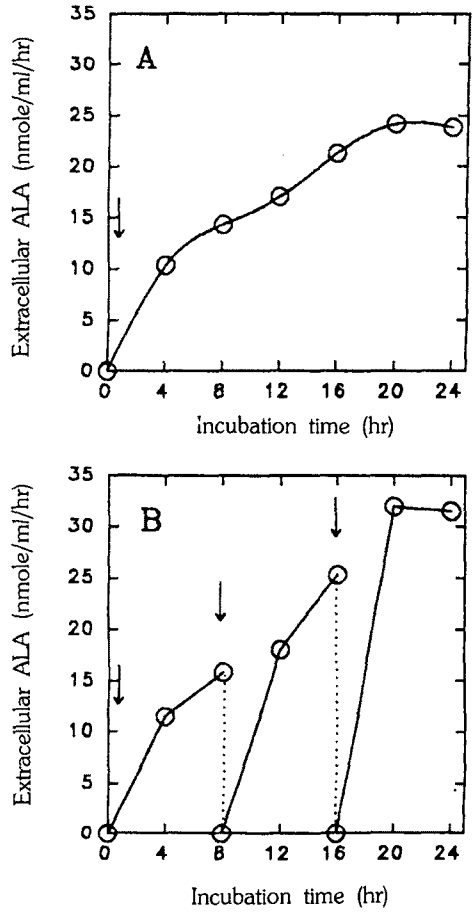

Figure 10. Increase in extracellular ALA formation by sequential feeding of L-glutamate during intact cell reaction.

The reactions were carried out by following procedures: (A), incubated in the presence of initial concentrations of 5 mM LA and 3 mM L-glutamate; (B), incubated in the presence of 1.5 mM LA and 1 mM L-glutamate up to the point indicated by 2nd arrow and the supernatant was harvested by centrifugation. The cells were then resuspended in the initial content, and post-incubation was followed. This process was repeated up to the last point of arrow and the entire yield of ALA by this method was compared with the maximum yield of ALA observed at 20 hr of incubation by single feed method (A).

compared to that produced by the single feeding system. At present, rational conditions for a successful production of extracellular ALA from the intact cell system are under investigation using the sequential feeding technique in the presence of cofactors which induce ALA production.

DISCUSSION

The rate of cell growth and reproduction in facultative autotrophs is often strictly dependent on the exogeneous

supply of nutrition, provided that the cells function tricarboxylic acid cycle (2). Such a mode was found in a strain of *Rhodocyclus gelatinosus* (13), a typical photoheterotroph, which requires a number of essential organic acids on growth. The use of growth-stalled intact cells prepared by this bacterial culture broth on ALA generation should therefore be conferring a great opportunistic fate of ALA precursors to be utilized effectively as materials not for cell growth but for selective ALA biosynthesis. Indeed, the above assumption was satisfied to some degree as can be seen from the data in Fig. 10 (B), which shows that the specific yield of extracellular ALA (maximum amount of ALA produced/concentration of precursors supplemented) obtained by a sequential feeding method was increased over 6-fold by using intact cells relative to that found in the vegetative culture method (Table 1). This finding is more ensured from the result that the initial purity of the extracellular ALA produced by the intact cells was not comparably high relative to that found in the classical cultural process. Meanwhile, the maximum concentration of extracellular ALA was observed by the use of intact cells within 20 hr of incubation while a period of over 5 days was required to meet the concentration when the cultural method was employed with this bacterium. These observations suggest that the newly proposed method by using the intact cells may be far more efficient in managing the ALA production process than the old method.

Levulinic acid (LA) has been known as a powerful inhibitor of ALA dehydratase activity, by which the intracellular concentration of ALA is increased (10). Although an unusual accumulation of ALA is a common phenomenon in the presence of this compound (26), it appears to be readily metabolized under the circumstance of the vegetative cell growth (8). A clear evidence confirming the above assumption was observed from the result shown in Fig. 1, indicating that the growth-retarded cells are required even less than one magnitude order of LA amount, to give the maximum yield of extracellular ALA relative to the amount used by the vegetative cells described in our previous report (7).

Although the novel use of intact cells proposed here is not satisfactory yet in view of the mass production of ALA, one can further speculate that by applicating the intact cells to a bioreactor system (6), if possible, in conjunction with the high ALA producing mutants the proposed method should be able to aid industrial application.

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