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Effect of the Supplement of Metabolites on Cell Growth and Poly-β-hydroxybutyrate Biosynthesis of *Alcaligenes latus*

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The characteristics of cell growth and poly-β-hydroxybutyrate biosynthesis of *Alcaligenes latus* ATCC 29713 were investigated. The PHB accumulation pattern of *A. latus* followed a growth-associated type where the cell growth and PHB accumulation were carried out simultaneously. Various intermediate compounds such as metabolites involved in the TCA cycle, amino acids, and saturated and unsaturated fatty acids were added to examine their effect on cell growth and PHB accumulation. Citrate, tyrosine, and palmitic acid showed the most significant increase both on cell growth and PHB accumulation. Maximum PHB concentrations were noticeably increased about 1.4 to 1.6 times higher than that of control, corresponding to 5.54, 6.45, and 6.45 g/l for citrate, tyrosine, and palmitic acid, respectively. The stimulatory effects of the supplemented metabolites were analyzed in terms of the increment of enzyme activities related to sugar catabolism and PHB biosynthesis.

Biodegradable poly-β-hydroxybutyrate (PHB) is typical of poly-β-hydroxyalkanoate (PHA), and is accumulated in the cytoplasms of various microorganisms, such as autotrophic or heterotrophic aerobic bacteria, anaerobic photosynthetic bacteria, gliding bacteria, *Actinomycetes*, and *Cyanobacteria* (1, 6, 12).

Alcaligenes eutrophus is typical of PHB producing bacterium that has been utilized recently for PHB production on a semi-commercial scale (23). PHB synthesis occurred actively under unbalanced growth conditions, where nitrogen, phosphate, and oxygen are limited, therefore, PHB production is performed using two-stage cultivation; growing of the cells during the first stage and then promoting the induction of PHB accumulation by maintaining unbalanced conditions during the second stage. Fed-batch cultivation, in which carbon is added stepwise to maintain unbalanced growth condition, is also used for PHB production.

Alcaligenes latus can not only accumulate PHB simultaneously in association with cell growth but also grows well on cheap carbon sources, such as sucrose, cane sugar, and beet sugar. Because of the above characteristics, *A. latus* is coming to notice as a promising strain for the production of PHB (5).

A. latus has been used as a production strain for PHB, and a semi-industrial scale process for PHB production using the mutant strain btF-96 only for one-stage cultivation has been developed (9, 11). Doi *et al.* (8, 21) also used *A. latus* for production of copolymers, P(3HB-*co*-3HP) and P(3HB-*co*-4HB), by the addition of 3-hydroxypropionate or γ-butyrolactone.

Maekawa *et al.* (16) suggested that the growth-associated characteristic of PHB biosynthesis in *A. latus* might be due to the relatively high rate of the condensation reaction of acetyl-CoA to acetoacetyl-CoA compared to the cleavage reaction of acetoacetyl-CoA to acetyl-CoA, the first step reaction related to PHB biosynthesis catalyzed by β-ketothiolase. As a consequence, the produced acetyl-CoA in various metabolic pathways can successfully flow into PHB biosynthesis even at the cell growth stage, unlike *A. eutrophus* (3, 16).

PHB biosynthesis is also controlled by the concentrations of intermediate metabolites, acetyl-CoA is used as the PHB precursor, and NADPH is used as a reducing power for PHB biosynthesis (15, 19). *A. latus* seems to have the burden of supply of acetyl-CoA and NADPH, because the aforementioned intermediates are utilized for both PHB biosynthesis and cell growth.

To enhance PHB biosynthesis in *A. latus*, therefore, an effective supply of acetyl-CoA and NADPH is a prerequisite. The addition of various intermediate compounds which consume acetyl-CoA and NADPH in their biosynthesis, such as the intermediate compounds of the TCA cycle, various amino acids, and fatty acids, can enhance

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PHB biosynthesis, because the supplement of the above-mentioned compounds may improve the availability of acetyl-CoA and NADPH used for PHB biosynthesis by reducing the requirement of acetyl-CoA and NADPH for metabolites biosynthesis other than PHB.

In this work, the characteristics of cell growth and PHB biosynthesis of *Alcaligenes latus* ATCC 29713 were investigated. The various intermediate compounds were added to increase the accumulation of PHB. The effect of supplements of citrate, palmitic acid, and tyrosine on the sugar metabolism and the PHB biosynthesis of *A. latus* were examined. Our observations will not only facilitate an understanding of the regulation mechanism of *A. latus* related to PHB biosynthesis but also help identify the proper cultivation conditions for the production of PHB.

MATERIALS AND METHODS

Strain and Culture Media

The strain used was *Alcaligenes latus* ATCC 29713. The minimal medium was composed of sucrose 10-20 g/l, Na_2HPO_4 8.6 g/l, KH_2PO_4 1.5 g/l, $(NH_4)_2SO_4$ 1.0 g/l, $MgSO_4$ 0.2 g/l, ammonium iron(III) citrate 0.06 g/l, $CaCl_2$ 0.01 g/l, and trace element solution (in 0.1N HCl: H_3BO_3 0.3 g/l, $CoCl_2 \cdot 6H_2O$ 0.2 g/l, ZnSO4 0.1 g/l, $MnCl_2 \cdot 4H_2O$ 0.03 g/l, $NaMoO_4$ 0.03 g/l, $NiCl_2 \cdot 6H_2O$ 0.02 g/l, and $CuSO_4 \cdot 5H_2O$ 0.01 g/l) 1 ml/l (8). The nutrient-rich medium was composed of polypepton 10 g/l, yeast extract 10 g/l, meat extract 5.0 g/l, and $(NH_4)_2SO_4$ 5.0 g/l. The strain preserved at -70°C after cultivation in the stock culture medium (10 g/l polypepton, 10 g/l yeast extract, 5.0 g/l meat extract, and 5.0 g/l NaCl), mixed with the same volume of 20% (w/w) glycerol, was activated in the nutrient-rich medium before cultivation.

Cultivation of Strain

Cultivation was carried out mainly in a flask shaker in the minimal medium at 30°C, and pH 7.0. The concentrations of carbon and nitrogen sources were changed in order to maintain a proper C/N ratio accordingly. The intermediate compounds of the TCA cycle (Sigma Co.) and amino acids (Sigma Co.) were prepared according to the method of Becker *et al.* (2), and then added to the minimal medium. The various saturated and unsaturated fatty acids of various concentrations (1-50 mM) were also added together with 0.1% (w/v) Brij35 as a surfactant.

Fermentor Cultivation

Fermentor cultivation was carried out in a 2.5 liter fermentor (Korea fermentor Co.) mainly in the minimal medium, with an inoculum size of 5.0% (v/v), 30°C, pH 7.0, an air flow rate of 1.0 vvm, and an agitation speed of 300 rpm. The strain was also cultivated after the supplements of citrate and tyrosine 60 mg/l, respectively, and palmitic acid 20 mM to examine the effect of metabolites on cell growth and PHB accumulation.

Measurements of Total and Residual Cell Mass

Total cell mass was determined after drying the harvested cells at 100°C for 24 h, and the residual cell mass was determined by subtracting PHB concentrations from the total cell mass.

Determination of PHB

PHB was isolated from cells according to the method of Law snd Slepecky (14) who used solvent for extraction. The PHB concentration was determined by the modified method of Braunegg and Bogensberger (3) using gas chromatography equipped with a flame ionization detector (Young-In Co. Ltd.). The analysis was carried out in gas column (6 m × 3 mm *i.d.*) filled with 2% Reoplex 400 on Chromosorb GAW 60 to 80 mesh, at a nitrogen flow rate of 30 ml/min, an initial temperature of 100°C, and a final temperature of 150°C. PHB powder of *A. eutrophus* (Sigma Co.) was used as a standard.

Analytical Methods

Sucrose concentration was determined by HPLC (Gilson Co.) with a Cosmosil $5NH_2$ packed column (Nacali Co.) using 0.01 N H_2SO_4 as the mobile phase. NH_4^+ concentration in the culture was measured by the modified phenolhypochlorite reaction method of Sonnleitner *et al.* (22) using $(NH_4)_2SO_4$ as a standard.

RESULTS AND DISCUSSION

Characteristics of Cell Growth and PHB Accumulation of *A. latus*

Patterns of cell growth and PHB accumulation. Fig. 1 illustrates the patterns of cell growth and PHB accumulation of *A. latus*, changes of the total cell mass, PHB, sucrose, and $(NH_4)_2SO_4$ concentrations, during cultivation in the minimal medium containing 20 g/l of sucrose and 2 g/l of $(NH_4)_2SO_4$. Cell started to grow actively from around 12 h after a relatively long lag period, and the most active cell growth was observed at around 24 h. The maximum total cell concentration of 6.32 g/l was achieved after 36 h, and then the total cell concentration started to decrease rapidly thereafter in contrast to *A. eutrophus*.

PHB accumulation also began at 12 h, and the most active PHB accumulation was observed at around 24 h at the phase where the most active cell growth occurred. The maximum PHB concentration of 4.02 g/l was achieved at around 36 h, about the same time as the total cell growth, and the PHB content was calculated to be 62.6%. The aforementioned patterns indicate that the PHB accumulation of *A. latus* can be classified as a growth-associated type, closely related with the cell growth, as previously reported by Braunegg and Bogensberger (3).

The PHB concentration in *A. latus* decreased drastically during the later stage of cultivation even though significant amounts of carbon remained in contrast to

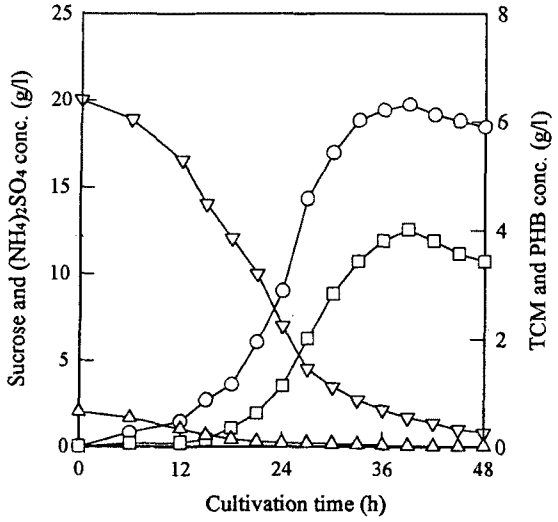

Fig. 1. Changes of cell growth and PHB accumulation during cultivation of *A. latus*.
Cultivation: minimal medium, 20 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, 2.5 l fermentor, pH 7.0, 30°C, and for 48 h. ○-○, Total cell mass (TCM); □-□, PHB conc.; ▽-▽, Sucrose conc.; △-△, $(NH_4)_2SO_4$ conc.

that of *A. eutrophus*. The observed high PHB consumption during the later stage means that *A. latus* probably has a much more active PHB mobilizing enzyme system as compared to *A. eutrophus*. The cell growth and PHB accumulation of *A. latus* ceased at the point where still significant amounts of carbon sources remained, and the reasons for this need to be clarified.

It has been reported that PHB dissipated at rather high rates in *P. oleovorans* and *P. aeruginosa* as compared to *A. eutrophus* at the stage where the carbon source was limited, and others explained this difference using the sizes of M. W. of PHAs of *Pseudomonas* sp. and that of *A. eutrophus* (7, 17). The M. W. of PHB of *A. latus*, 3.50×10^5, is around a half of that of *A. eutrophus*, 7.37×10^5 (8, 12). Therefore, the observed high PHB consumption rate in *A. latus* as compared to *A. eutrophus* may be explained by the differences in M. W. of PHB, however, further study needs to be conducted to clarify the function of the PHB-mobilizing enzyme system.

Effect of C/N ratio. To investigate the influence of the C/N ratio on cell growth and PHB accumulation, *A. latus* was cultivated in the nutrient rich medium during the first stage, and then it was recultivated after separation at the second stage in the minimal medium containing different amounts of $(NH_4)_2SO_4$ from 0.01 to 10.0 g/l but with a fixed sucrose concentration of 20 g/l. Table 1 compares the changes in profiles of total cell mass, PHB, and PHB content as measured after 48 h.

The PHB concentration in *A. latus* was increased as the C/N ratio decreased, in contrast to *A. eutrophus*. The declined PHB concentration in *A. latus* at the higher C/N ratio, in spite of the high PHB content, is caused by the growth limitation of cells caused by the deficiency of nitrogen. Maximum total cell and PHB concentrations, corresponding to 8.17 and 4.42 g/l, respectively, were obtained at the C/N ratio of 6.6.

Table 1. Effect of C/N ratio on cell growth and PHB accumulation of *A. latus* at two-stage cultivation.

$(NH_4)_2SO_4$ (g/l)	C/N ratio	TCM[1] (g/l)	PHB (g/l)	Content[2] (%)
0.00	—	0.84	0.75	89.2
0.01	200	0.94	0.81	86.1
0.50	40	2.87	2.16	75.4
1.00	20	5.27	3.18	60.3
3.00	6.6	8.17	4.42	54.1
5.00	4.0	7.20	3.08	42.8
10.00	2.0	2.56	0.92	35.9

Cultivation: harvested cells from first stage, two-stage cultivation, minimal medium, 20 g/l of sucrose, 0.01-10 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 48 h. [1]total cell mass; [2](PHB/TCM)×100.

Table 2. Effect of the supplement of the TCA cycle intermediates on cell growth and PHB accumulation of *A. latus*.

TCA cycle intermediates (40 mg/l)	TCM[1] (g/l)	PHB (g/l)	Content[2] (%)	$Y_{P/S}$[3] (g/g)
No addition	4.93	2.92	59.2	0.322
Citrate	5.48	4.08	74.4	0.421
Isocitrate	5.28	3.89	73.6	0.409
Glyoxalate	4.82	3.29	68.2	0.348
Oxoglutarate	4.36	2.78	63.7	0.289
Succinate	4.12	2.91	70.6	0.319
Fumarate	5.01	3.13	42.5	0.357
Malate	4.01	1.98	49.3	0.232
Oxaloacetate	5.13	3.62	70.6	0.387

Cultivation: minimal medium, 10 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 36 h, and supplemented with 40 mg/l of intermediates. [1]total cell mass; [2](PHB/TCM)×100; [3](g/l of PHB)/(g/l of sucrose consumed).

The carbon source is utilized mainly for PHB accumulation at a high C/N ratio in *A. eutrophus*, because of the nongrowth-associated PHB accumulation pattern. Therefore, to achieve efficiency of PHB accumulation, *A. latus* needs to be cultivated under conditions where active cell growth can occur rather than unbalanced growth conditions to induce PHB synthesis by inhibiting cell growth, unlike *A. eutrophus*.

Effect of the Supplement of Intermediate Compounds of the TCA Cycle on Cell Growth and PHB Biosynthesis of *A. latus*

Intermediate compounds. In order to examine the effect of the supplement of intermediate compounds of the TCA cycle, *A. latus* was cultivated in a minimal medium containing 10 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, and a supplement of 40 mg/l of various TCA cycle related compounds. Table 2 compares the concentrations

of total cell mass, PHB, and PHB yield per an amount of sucrose utilized as measured after cultivation for 36 h. Among them, the supplement of citrate, isocitrate, and glyoxalate showed a substantial increase in the total cell mass and PHB accumulation, while other intermediate compounds did not show any significant change.

The above substances are compounds related to citrate biosynthesis in the TCA cycle during which acetyl-CoA is consumed. The addition of the aforementioned metabolites seems to increase the flow of acetyl-CoA to PHB biosynthesis either by reducing the consumption of acetyl-CoA for citrate biosynthesis or by decreasing the efficiency of the TCA cycle by the inhibitory effect of above intermediary compounds. The product inhibitory effect of citrate on citrate synthetase has been reported recently (20). Citrate showed the best results, and total cell concentration, PHB concentration, and PHB yield per an amount of sucrose utilized were 5.48 g/l, 4.08 g/l, and 0.421, respectively.

Citrate concentration. Fig. 2 shows the effect of citrate concentrations ranging from 10 to 100 mg/l after 36 h. The PHB accumulation was increased as the citrate concentration was augmented up to 60 mg/l, and the maximum concentrations of total cell and PHB, and PHB content were 5.84 g/l, 4.56 g/l, and 78.0%, respectively. Meanwhile, the residual cell growth and total cell mass after subtracting PHB concentration, were not at all influenced by the supplement of citrate. Therefore the addition of a small amount of citrate as a PHB biosynthesis activator seems to be an effective method to increase PHB accumulation in *A. latus*.

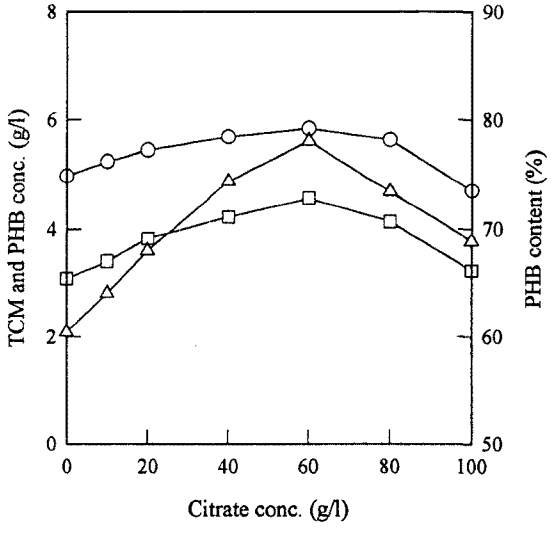

Fig. 2. Effect of citrate concentration on cell growth and PHB accumulation of *A. latus*.
Cultivation: minimal medium, 20 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 36 h, and supplemented with 10-100 mg/l of citrate. ○-○, Total cell mass (TCM); □-□, PHB conc.; △-△, PHB content.

However, at a concentration higher than 80 mg/l, the PHB accumulation was decreased because the excessive addition of citrate seemed to drastically decrease the function of the metabolic pathway related to cell growth, for example the activity of pyruvate kinase of the glycolysis and citrate synthetase of the TCA cycle.

Effect of Saturated and Unsaturated Fatty Acid on Cell Growth and PHB Accumulation of *A. latus*

Saturated and unsaturated fatty acids. *A. latus* was cultivated in a minimal medium containing 10 mM of saturated long chain fatty acids from caproic acid (C_6) to steric acid (C_{18}), and the total cell mass and the PHB concentration were measured after 60 h as summarized in Table 3. In medium chain fatty acid less than C_{14}, PHB was not accumulated because the cell could not grow well with the low carbon number fatty acid. However, as the chain length increased, both cell growth and PHB accumulation started to increase, and the best stimulatory effect on PHB accumulation was achieved with palmitic acid whose chain length is C_{16}.

Fad regulon that regulates the biosynthesis of enzymes related to the β-oxidation in *E. coli* has been studied by Weeks *et al.* (25), and they reported that *fad* regulon was induced by long chain fatty acid, but not by a medium chain fatty acid. Therefore it can be assumed that *A. latus* may follow a similar regulation mechanism of *E. coli*, but further study needs to be conducted.

Long chain fatty acids seem to be utililized directly as the carbon sources for cell growth and PHB accumulation through the β-oxidation reaction. For palmitic acid, the maximum concentrations of total cell mass and PHB were measured to be 6.18 g/l and 4.14 g/l.

The effect of the supplement of unsaturated long fatty acids from myristoleic acid (*cis*-9-tetradecenoic acid) of C_{14} to eicosenoic acid (*cis*-1-eicosenoic acid) of C_{20} on cell growth and PHB accumulation were also in-

Table 3. Effect of the supplement of saturated fatty acid on PHB accumulation of *A. latus*.

Saturated fatty acid (10 mM)	TCM[1] (g/l)	PHB (g/l)	Content[2] (%)	$Y_{P/S}$[3] (g/g)
No addition*	4.98	2.89	58.0	0.322
Caproic acid	ND	ND	ND	ND
Caprylic acid	ND	ND	ND	ND
Capric acid	ND	ND	ND	ND
Lauric acid	0.26	ND	ND	ND
Myristic acid	2.04	0.98	48.0	0.298
Palmitic acid	6.18	4.14	66.9	0.421
Magaric acid	5.21	3.46	66.4	0.386
Stearic acid	5.01	3.22	64.2	0.343

Cultivation: minimal medium, 10 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 60 h, and supplemented with 10 mM of saturated fatty acids. [1]total cell mass; [2](PHB/TCM)×100; [3](g/l of PHB)/(g/l of sucrose consumed). * after 48 h.

vestigated as shown in Table 4. *A. latus* can not grow well with the unsaturated fatty acids unlike the saturated fatty acid, indicating that *A. latus* can not utilize fatty acids containing one more double bond.

Concentration of palmitic acid. Fig. 3 shows the effect of palmitic acid concentrations ranging from 1 to 100 mM after 60 h. As palmitic acid increased from 1 to 30 mM, total cell mass and PHB concentrations were increased, and the maximal concentrations were obtained at 20 mM of palmitic acid, corresponding to 6.41 g/l and 4.36 g/l, respectively. This enhancement can be explained by the increased availability of acetyl-CoA and NAD(P)H that are generated through β-oxidation.

Effect of the Supplement of Amino Acid on Cell Growth and PHB Accumulation

Amino acids. In order to investigate the effect of supplementing various amino acids, *A. latus* was cultivated in a minimal medium supplemented with 40 mg/l of various amino acids. Table 5 compares the total cell mass, PHB concentration, and PHB yield of the sucrose utilized in the minimal medium supplemented with 40 mg/l of various amino acids after cultivation for 36 h.

The total cell mass and the PHB concentration were influenced significantly by most of the amino acids, especially by leucine, glutamine, and tyrosine. Among amino acids, tyrosine showed the most significant effect, and the total cell mass, PHB concentration, and PHB yield of sucrose utilized were measured to be 5.68 g/l, 4.65 g/l, and 0.468, respectively.

Lee *et al.* (15), who studied PHB production by a recombinant *E. coli* containing cloned PHB biosynthesis gene, reported that the additions of cysteine, isoleucine, and threonine result in significantly increased PHB accumulation. They proposed that the enhancement of PHB accumulation could be due to the increased availability of acetyl-CoA and NADPH due to the addition of the above amino acids which required a relatively high number of NADPH and acetyl-CoA for their biosynthesis.

Table 4. Effect of the supplement of unsaturated fatty acid on PHB accumulation.

Unsaturated fatty acid (10 mM)	TCM[1] (g/l)	PHB (g/l)	Content[2] (%)	$Y_{P/S}$[3]
No addition*	4.98	2.89	58.0	0.322
Myristoleic acid	1.20	0.51	42.5	0.251
Palmitoleic acid	3.01	1.21	40.1	0.211
Oleic acid	3.68	1.79	48.6	0.279
cis-Vaccenic acid	3.16	1.25	39.5	0.225
Linoleic acid	2.03	0.94	44.8	0.094
Eicosenoic acid	1.1	0.63	57.2	0.063

Cultivation: minimal medium, 10 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 60 h and supplemented with 10 mM of unsaturated fatty acids. [1]total cell mass; [2](PHB/TCM)×100; [3](g/l of PHB)/(g/l of sucrose consumed). *after 48 h.

The improved results for cell growth and PHB accumulation of *A. latus* were obtained by the additions of leucine, glutamine, and tyrosine, which required less NADPH for their biosynthesis as compared to other amino acids, cysteine, isoleucine, and threonine. The supplement of amino acids during a cultivation of *A. latus* may reduce the consumption of acetyl-CoA and NADPH used for amino acid biosynthesis, as a consequence,

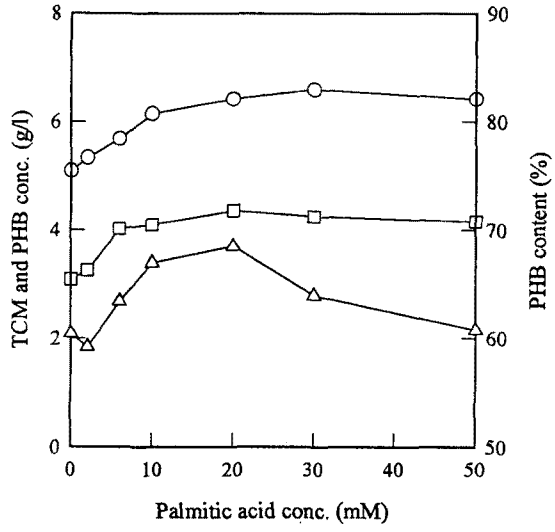

Fig. 3. Effect of palmitic acid concentration on cell growth and PHB accumulation of *A. latus*.
Cultivation: minimal medium, 20 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 60 h, and supplemented with 2-50 mM of palmitic acid. ○-○, Total cell mass (TCM); □-□, PHB conc.; △-△, PHB content.

Table 5. Effect of the supplement of amino acids on cell growth and PHB accumulation of *A. latus*.

Amino acid (40 mg/l)	TCM[1] (g/l)	PHB (g/l)	Content[2] (%)	$Y_{P/S}$[3]
No addition	4.93	2.92	58.0	0.322
Ala	5.39	3.88	70.6	0.398
Arg	5.20	3.94	74.3	0.418
Asp	4.27	3.49	81.7	0.369
Cys	4.72	2.91	61.6	0.341
Gln	5.46	4.12	74.1	0.422
Glu	5.31	3.98	73.5	0.415
Gly	5.39	3.69	67.2	0.389
His	4.89	3.91	79.9	0.426
Ile	4.94	3.81	77.1	0.401
Leu	5.37	4.24	78.9	0.436
Phe	5.08	3.50	67.5	0.378
Ser	5.34	3.23	59.3	0.343
Thr	4.98	3.72	74.6	0.390
Tyr	5.68	4.58	81.8	0.468

Cultivation: minimal medium, 10 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 36 h, and supplemented with 40 mg/l of amino acids. [1]total cell mass; [2](PHB/TCM)×100; [3](g/l of PHB)/(g/l of sucrose consumed).

acetyl-CoA and NADPH can flow more easily to PHB biosynthesis, however, further study needs to be carried out.

Tyrosine concentration. Fig. 4 compares the effect of tyrosine concentrations ranging from 10 to 100 mg/l, on the total cell growth and PHB accumulation. The total cell mass and the PHB concentration increased, as the tyrosine concentration increased up to 60 mg/l, and the maximal concentrations of total cell and PHB were achieved at 60 mg/l of tyrosine concentration, corresponding to 5.68 g/l and 4.58 g/l, respectively. Especially, the PHB content increased drastically upon with the addition of tyrosine from 62.8% to 80.6%. The supplement of adequate tyrosine seems to make the activity of aromatic amino acid biosynthesis decreased by the feedback inhibition of tyrosine, therefore the PHB accumulation was enhanced by increasing the flows of acetyl-CoA and NADPH to PHB biosynthesis.

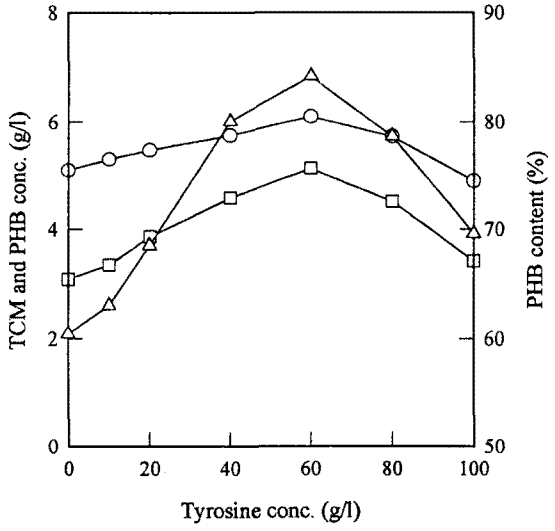

Fig. 4. Effect of tyrosine concentration on cell growth and PHB accumulation of *A. latus*.
Cultivation: minimal medium, 20 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, pH 7.0, 30°C after 36 h, and supplemented with 10-100 mg/l of tyrosine. ○-○, Total cell mass (TCM); □-□, PHB conc.; △-△, PHB content.

Cultivation of *A. latus* in the Presence of Citrate, Palmitic acid, and Tyrosine

Supplement of citrate. Fig. 5 (A, B, and C) illustrates the profiles of total cell mass, PHB, and sucrose concentrations during the cultivation of *A. latus* in a minimal medium supplemented with 60 mg/l of citrate (A), 60 mg/l of tyrosine (B), and 20 mM of palmitic acid (C), respectively. As shown in Fig. 5 (A), the growth rate was increased substantially, the maximum total cell concentration, PHB concentration, and PHB content were increased from 6.32 to 7.24 g/l, from 4.02 to 5.54 g/l, and from 62.6 to 75.4% as compared to those of the control illustrated in Fig. 1.

The sugar consumption rate was also increased substantially by the supplement of citrate as can be seen in Fig. 5 (A), meanwhile, a substantial amount of the residual sucrose corresponding to 2.12 g/l remained in the medium even after 36 h as shown in Fig. 1. The increased sucrose utilization makes it possible for the added carbon source to be used for total cell growth and PHB accumulation, hence the increased PHB yield for sucrose was obtained. This indicates that the activity of sugar catabolism can be enhanced by the addition of citrate, a stimulator of PHB biosynthesis.

Supplement of tyrosine. As shown in Fig. 5 (B), the cell growth and PHB accumulation increased similarly with that observed when citrate was supplemented. However the effect of tyrosine was much more critical as compared to the citrate supplement. The maximal concentrations of total cell mass and PHB were 7.86 g/l and 6.45 g/l, respectively, and the rate of sucrose utilization

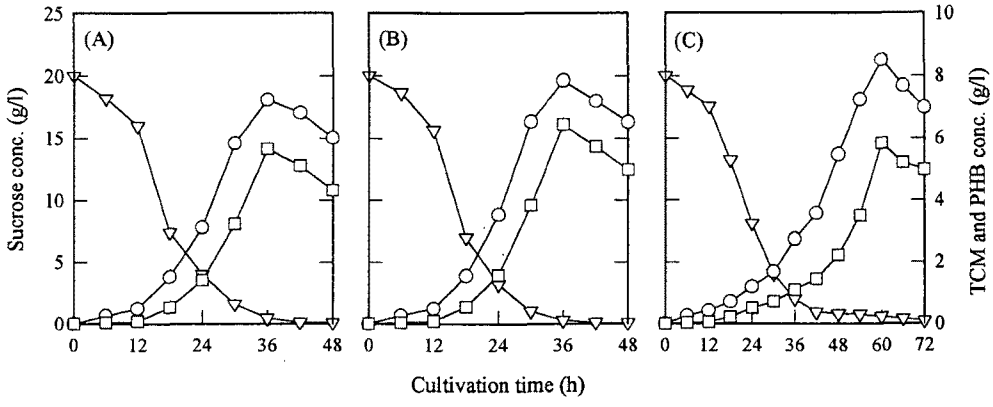

Fig. 5. Cultivation of *A. latus* by supplement of citrate (A), tyrosine (B), and palmitic acid (C).
Cultivation: minimal medium, 20 g/l of sucrose, 2 g/l of $(NH_4)_2SO_4$, 25 l fermentor, pH 7.0, 30°C, (A) and (B) for 48 h, and (C) for 72 h. ○-○, Total cell mass (TCM); □-□, PHB conc.; ▽-▽, Sucrose conc.

was also increased by the tyrosine supplement as compared to citrate.

Supplement of palmitic acid. Cell growth proceeded more slowly up to 48 h as compared to the control as shown in Fig. 1 where sucrose was nearly exhausted, however, the cell growth continued thereafter up to 60 h because the palmitic acid began to be utilized as a carbon source, and the final total cell concentration was 8.01 g/l. The cell growth pattern showed typical diauxie growth with a short lag phase from 42 to 48 h because of catabolite repression by two competing carbon sources, sucrose and palmitic acid. The maximum PHB concentration was 5.51 g/l, and the PHB content was calculated to be 68.7%, a lower value as compared to those of citrate and tyrosine.

This may be due to the relatively low PHB induction effect of palmitic acid because the NADH, which is utilized for residual cell growth, is mainly generated through the β-oxidation of palmitic acid, instead of NADPH which is used as a reducing power for PHB biosynthesis.

The enhancement of PHB concentrations cannot be explained by a direct contribution of the supplemented intermediates on PHB biosynthesis because the amount of intermediate compounds added, around 60 mg/l each of citrate and tyrosine, is too small an amount to be used as the building block of PHB. Therefore it can be assumed that the added intermediates may affect mainly on the various enzyme activities in *A. latus*, such as glycolysis, the TCA cycle, and PHB biosynthesis. It can be postulated that citrate may directly influence citrate synthetase in the TCA cycle and tyrosine may influence glucose-6-phosphate dehydrogenase in glycolysis (10), those are the key regulatory enzymes which control the intracellular concentrations of acetyl-CoA and NADPH, and therefore PHB biosynthesis. However, further research to clarify the effects of the supplement of the above intermediate compounds on enzyme activities needs to be conducted.

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