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Enzymatic Characteristics of Biosynthesis and Degradation of Poly-β-hydroxybutyrate of *Alcaligenes latus*

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The enzymatic characteristics of *Alcaligenes latus* were investigated by measuring the variations of various enzyme activities related to biosynthesis and degradation of poly-β-hydroxybutyrate (PHB) during cultivation. All PHB biosynthetic enzymes, β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, were activated gradually at the PHB accumulation stage, and the PHB synthase showed the highest value among three enzymes. This indicates that the rate of PHB biosynthesis is mainly controlled by either β-ketothiolase or acetoacetyl-CoA reductase rather than PHB synthase. The enzymatic activities related to the degradation of PHB were also measured, and the degradation of PHB was controlled by the activity of PHB depolymerase. The effect of supplements of metabolic regulators, citrate and tyrosine, was also investigated, and the activity of glucose-6-phosphate dehydrogenase was increased by metabolic regulators, especially by tyrosine. The activities of β-ketothiolase and acetoacetyl-CoA reductase were also activated by citrate and tyrosine, while the activity of PHB depolymerase was depressed. The increased rate and yield of PHB biosynthesis by metabolic regulators may be due to the increment of acetyl-CoA concentration either by the repression of the TCA cycle by citrate through product inhibition or by the activation of sucrose metabolism by the supplemented tyrosine.

Poly-β-hydroxybutyrate (PHB) is an intracellular energy storage material accumulated by many microorganisms under abnormal growth conditions (1, 8). *Alcaligenes latus* can accumulate PHB exceptionally well more than 60% even without enforcing the nutritional limitation, therefore, it has been recognized as a potential industrial strain for PHB production (6).

The PHB biosynthesis and its regulation mechanism have been mainly carried out using *Alcaligenes eutrophus* (8) and *Azotobacter beijerinckii* (18). The biosynthesis of PHB from intermediate acetyl-CoA is carried out by a sequence of three PHB biosynthetic enzymes: β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase. It has been reported that the key regulatory enzymes for PHB biosynthesis in *A. eutrophus* and *A. beijerinckii* are two; one is β-ketothiolase which catalyzes the first step of PHB biosynthesis and the other is glucose-6-phosphate dehydrogenase which supplies NADPH as the reducing power required for the second step of PHB biosynthesis (8, 17).

Meanwhile, the accumulated PHB is degraded to acetoacetyl-CoA by the three sequential intracellular PHB degradation enzymes: PHB depolymerase, D-(-)-3-hydroxybutyrate dehydrogenase, and acetoacetyl-CoA synthetase. The conversed acetoacetyl-CoA is broken down to acetyl-CoA by β-ketothiolase (17). The two enzymes, PHB depolymerase which initiates PHB degradation and D-(-)-3-hydroxy-butyrate dehydrogenase catalyzing the first step of energy generation, seem to be the most critical enzymes for PHB degradation of above strains. It has been reported that PHB synthesis and degradation occurred simultaneously under normal growth condition (7). Therefore, to achieve an effective accumulation of PHB, the activities of enzymes related to PHB biosynthesis need to be induced, while those related to PHB degradation be depressed.

The research works on *A. latus* have been mainly focused on the cultivation of a strain to increase the rate and yield of PHB (6, 9). The apprehension of the enzymatic characteristics of *A. latus* according to cultivation conditions is essential not only for understanding the regulation mechanism but also for establishing the proper cultivation conditions, however, no intensive research works have been carried out on this aspect. Only Maekawa *et al.* (14) have purified β-ketothiolase to investigate the enzyme kinetics.

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In our previous work (13), the characteristics of cell growth and PHB biosynthesis of *A. latus* ATCC 29713 were investigated. Various metabolic regulators, including various intermediates of the tricarboxylic acid (TCA) cycle, amino acids, and fatty acids, were supplemented to increase the cell growth and PHB biosynthesis in *A. latus*. Citrate, tyrosine and palmitic acid, among the intermediates of the TCA cycle, amino acids, and fatty acids, respectively, showed the most significant effects both on cell growth and PHB accumulation.

In this work, the various enzyme activities related to biosynthesis and degradation of PHB, such as β-ketothiolase, acetoacetyl-CoA reductase, PHB synthase, PHB depolymerase and D-(-)-3-hydroxybutyrate dehydrogenase, along with glucose-6-phosphate dehydrogenase, were measured to understand the enzymatic characteristics of *A. latus* for PHB accumulation. The effect of supplements of metabolic regulators, citrate and tyrosine, on the activities of the above enzymes were also investigated. This is essential knowledge not only for establishing strategy for strain improvement but also for understanding the regulation mechanisms of PHB biosynthesis and degradation of *A. latus*.

MATERIALS AND METHODS

Strain

The strain was *Alcaligenes latus* ATCC 29713 studied previously in our laboratory (13).

Culture Media

The minimal medium (per liter) was composed of 10-20 g of sucrose, 8.6 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 0.06 g of ammonium iron (III) citrate, 0.01 g of $CaCl_2$, and 1 ml of microelement solution (8). The nutrient-rich medium (per liter) was composed of 10.0 g of polypepton, 10.0 g of yeast extract, 5.0 g of meat extract, and 5.0 g of $(NH_4)_2SO_4$, used for activation of strain preserved at -70°C.

Cultivation

The flask cultivation of *A. latus* was mainly carried out in the minimal medium at pH 7.0 and 30°C. It was also cultivated after the supplement of each of citrate and tyrosine prepared by the method of Becker *et al.* (4) at each concentration of 60 mg/ml. Fermentor cultivation was carried out in a 2.5 liter fermentor (Korea Fermentor Co., Incheon, Korea) under the cultivation conditions of inoculum size of 5.0% (v/v), air flow rate of 1.0 vvm, 30°C, pH 7.0, and 300 rpm.

Measurements of Total and Residual Cell Mass Concentrations

The total cell mass was determined after drying the cells at 100°C, and the residual cell mass was obtained by subtracting PHB concentration from the total cell mass.

Determination of PHB

The extracted PHB in hot chloroform after treatment of cells with 5% sodium hypochlorite solution at 37°C for 1 h was precipitated with hexane, and then filtered for purification (12). The PHB concentration was determined by gas chromatography (GC) using the modified method of Braunegg and Bogensberger (3, 13). The GC (Young-In Co. Ltd., Seoul, Korea) was composed of the flame ionization detector and a gas column (6 m × 3 mm) filled with 2% Reoplex 400 on Chromosorb GAW 60 to 80 mesh. The analysis was carried out at a nitrogen flow rate of 30 ml/min, an initial temperature of 100°C, and a final temperature of 150°C, using PHB powder of *A. eutrophus* (Sigma Co., St. Louis, MO, USA) as a standard.

Measurement of Enzyme Activities

To measure the various activities of enzymes, the cells were disrupted after being suspended in 50 mM phosphate buffer by ultrasonication at 4°C to obtain cell extract. The specific activities of various enzymes in the cell extract were determined by measuring the concentration of corresponding detection materials at different wavelengths after incubation with the appropriated substrates as summarized in Table 1.

In addition the activity of PHB depolymerase was measured by the method of Cho *et al.* (5) after modification as follows. Cells were cultivated in the minimal medium containing fructose and radioactive D-(1-^{14}C) fructose (Amersham Co., Buckingshaire, U.K.) at the ratio of 1000 to 1 for 48 h. 10 mg of the purified ^{14}C-labelled PHB (12,000 cpm/mg) was suspended in 0.1 M Tris-HCl buffer (pH 7.0), mixed with cell extract, and then incubated at 30°C for 30 min. The released radioactivity of ^{14}C-labelled β-hydroxybutyrate was measured using liquid scintillation analyzer (Packard Instrument Co., Inc., IL, U.S.A.).

Table 1. Methods used for determination of various enzyme activities.

Enzyme	Wavelength employed, nm	Detected material	Reference
β-Ketothiolase	340	NADH	Senior *et al.* (5)
Acetoacetyl-CoA reductase	340	NADPH	Senior *et al.* (5)
PHB synthase	412	CoA	Senior *et al.* (5)
PHB depolymerase	–	^{14}C-β-hydroxybutyrate	Cho *et al.* (13)
β-Hydroxybutyrate dehydrogenase	340	NADH	Senior *et al.* (4)
Glucose-6-phosphate dehydrogenase	340	NADPH	Senior *et al.* (5)

Analytical Methods

Sucrose concentration was determined by HPLC (Gilson Medical Electronics Inc., Villiers-le-Bel, France) with a Cosmosil $5NH_2$ packed column (Nacalai Tesque Inc., Tokyo, Japan), 0.01 N H_2SO_4 as the mobile phase. Protein concentration was measured by the method of Bradford (2) using bovine serum albumin (Sigma Co., St. Louis, MO, USA) as a standard.

RESULTS AND DISCUSSION

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

Fig. 1 illustrates the changes of total and residual cell mass, PHB, and sucrose concentration of *A. latus* during cultivation in the minimal medium containing sucrose 20 g/l and $(NH_4)_2SO_4$ 2 g/l. The patterns of total cell mass and PHB concentrations indicates that *A. latus* is following the typical growth-associated PHB accumulation pattern as observed previously (13).

Another characteristic was the rapid degradation of the accumulated PHB compared to *A. eutrophus* from the stationary growth phase even where a carbon source was available. The maximum PHB degradation rate of *A. latus* was calculated to be 0.082 g/l/h which was a very high value compared to the 0.025 g/l/h of *A. eutrophus* obtained by Kawaguchi and Doi (10) who also investigated the kinetics of PHB degradation. The rapid degradation of PHB in *A. latus* may be explained either by the low molecular weight of PHB granules accumulated in *A. latus* or by the well developed function of the PHB degradation enzyme system.

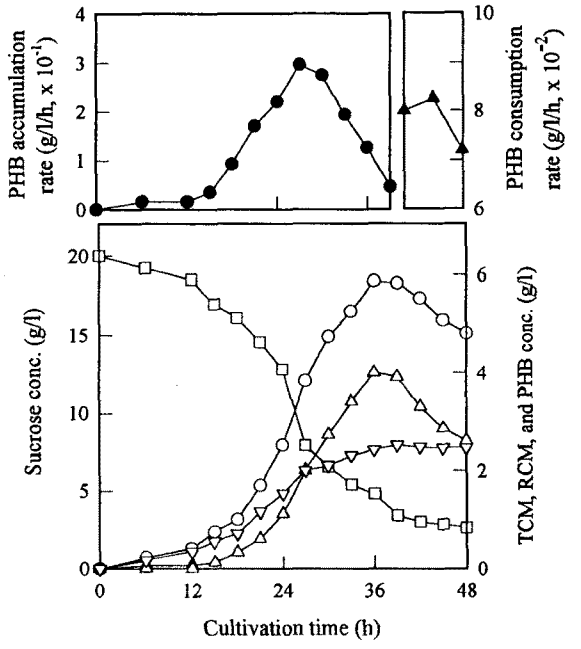

Fig. 1. Characteristics of cell growth and PHB accumulation of *A. latus*.
Cultivation: Minimal medium (per liter), 20 g of sucrose, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 48 h. ○, total cell mass (TCM); △, PHB concentration; ▽, residual cell mass (RCM); □, sucrose concentration; ●, PHB accumulation rate; ▲, PHB comsumption rate.

Variations of Specific Activities of PHB Biosynthesis and Degradation Enzymes in *A. latus* during Cultivation

PHB biosynthetic enzymes. Fig. 2 illustrates the changes of the specific activities of three PHB biosynthetic enzymes; β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase during cultivation. The specific activities of the three PHB biosynthetic enzymes were increased gradually up to 30 h where PHB accumulation occurred actively and accorded well with the accumulation pattern of PHB. The maximum specific activities of the three PHB biosynthetic enzymes were achieved at around 24 h, and measured to be 0.664, 0.714 and 8.736 units/mg protein, respectively.

The specific activity of PHB synthase showed the highest value, being ten times higher than those of β-ketothiolase and acetoacetyl-CoA reductase which both showed similar values. This suggests that the rate of biosynthesis of PHB in *A. latus* may be controlled by either β-ketothiolase or acetoacetyl-CoA reductase showing the lower values not by the PHB synthase, this is similar with *A. eutrophus* (14).

During the cultivation period where PHB accumulation ceased, all specific activities related to PHB biosyn-

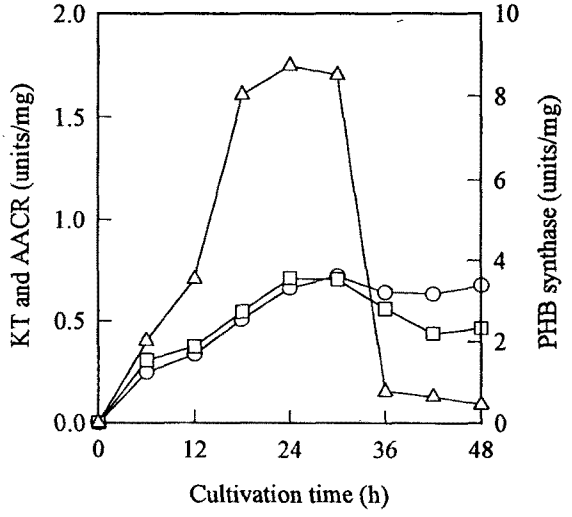

Fig. 2. Variations of the specific activities of β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase of *A. latus* during cultivation.
Cultivation: Minimal medium (per liter), 20 g of sucrose, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 48 h. ○, β-ketothiolase (KT); □, acetoacetyl-CoA reductase (AACR); △, PHB synthase.

thesis started to decrease, particularly PHB synthase decreased drastically and remained at a low level thereafter. On the other hand, the specific activity of β-ketothiolase remained at a comparatively higher value. The higher level of residual activity of β-ketothiolase can be explained by the special dual functions of β-ketothiolase in that it participates not only in PHB biosynthesis but also in the PHB degradation reaction that converses acetoacetyl-CoA to acetyl-CoA for starting material for the TCA cycle.

PHB degradation enzymes. Fig. 3 illustrates the specific activities of two critical enzymes related to PHB degradation; PHB depolymerase and β-hydroxybutyrate dehydrogenase during cultivation. The specific activity of PHB depolymerase, the first step enzyme of PHB degradation that breaks down PHB to D-(-)-3-hydroxybutyrate, showed a high value even during the early cultivation period, corresponding to 518.30 relative activity/mg protein. This initial high value of PHB depolymerase can be explained by the physiological state of *A. latus* that has to utilize PHB as an energy sources by degradation of PHB before consumption of sucrose. The PHB depolymerase decreased thereafter steadily until 24 h where PHB accumulation occurred actively, however, it increased again as the PHB biosynthesis declined.

On the other hand, the specific activity of β-hydroxybutyrate dehydrogenase, the second enzyme which generates NADH from the conversion of D-(-)-3-hydroxybutyrate to acetoacetate, showed a gradually increasing pattern throughout the cultivation period in contrast to that of PHB depolymerase. Page and Manchak (15) who studied the regulation mechanism of biosynthesis of polyhydroxyalkanoate in *Azotobacter vinelandii* also observed a similar trend for the activity of β-hydroxybutyrate dehydrogenase.

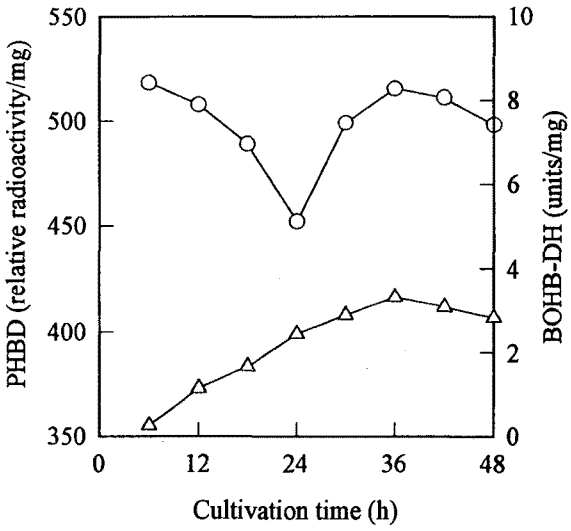

Fig. 3. Variations of the specific activities of PHB epolymerase and β-hydroxybutyrate dehydrogenase of *A. latus* during cultivation.
Cultivation: Minimal medium (per liter), 20 g of sucrose, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 48 h. ○, PHB depolymerase (PHBD); △, β-hydroxybutyrate dehydrogenase (BOHB-DH).

Steinbüchel *et al.* (19), who studied the regulation mechanism of PHB degradation enzymes in *A. eutrophus*, have reported that PHB degradation enzymes, both PHB depolymerase and β-hydroxybutyrate dehydrogenase, are controlled by the carbohydrate transport system, more specifically by phosphoenolpyruvate: carbohydrate phosphotransferase (PTS) system, as a consequence, PHB degradation is repressed when a carbon source is plentiful, while it is activated when carbon is limited. PHB depolymerase of *A. latus* studied in this work also seems to be controlled by a similar regulation mechanism to *A. eutrophus*. However in *A. latus*, β-hydroxybutyrate dehydrogenase may not be controlled by the PTS system considering the activity level increased gradually during cultivation in contrast to *A. eutrophus* (14).

The degradation of PHB in *A. latus* can be repressed to provide proper regulation of PHB depolymerase if imposed during cultivation. The activity of PHB depolymerase is closely related to the PTS system for sucrose transport, as a consequence, the degradation of PHB can be overcome by repressing the action of PHB depolymerase by maintaining sucrose concentrations at a properly low level, i.e. 3.24-4.65 g/l (6), during cultivation using fed-batch or continuous culture methods.

Effect of Supplements of Citrate and Tyrosine on Cell Growth and PHB Biosynthesis of *A. latus*

Fig. 4 illustrates the profiles of total cell mass, PHB, and sucrose concentration of *A. latus* cultivated in the minimal medium containing sucrose as a carbon source (A), and supplemented with citrate (B) and tyrosine (C) at 60 mg/l, respectively. Cell growth and PHB accumulation increased substantially after the supplement of citrate and tyrosine, and tyrosine showed the better result. The sucrose consumption rate was increased substantially, and the sucrose was more completely utilized, compared to the control where residual sucrose remained at concentration of 2.72 g/l even after 48 h.

The supplemented citrate can be used as a starting material for PHB biosynthesis through the lysis reaction of citrate lyase, and tyrosine can be used for the supply of NADPH as a reducing power for PHB biosynthesis through the oxidation reaction of amino acid dehydrogenase. To understand the effect of the metabolic regulators on cell growth and PHB biosynthesis, the various enzyme activities related to sucrose catabolism, and biosynthesis and degradation of PHB have to be investigated.

Effect of Supplement of Metabolic Regulators on

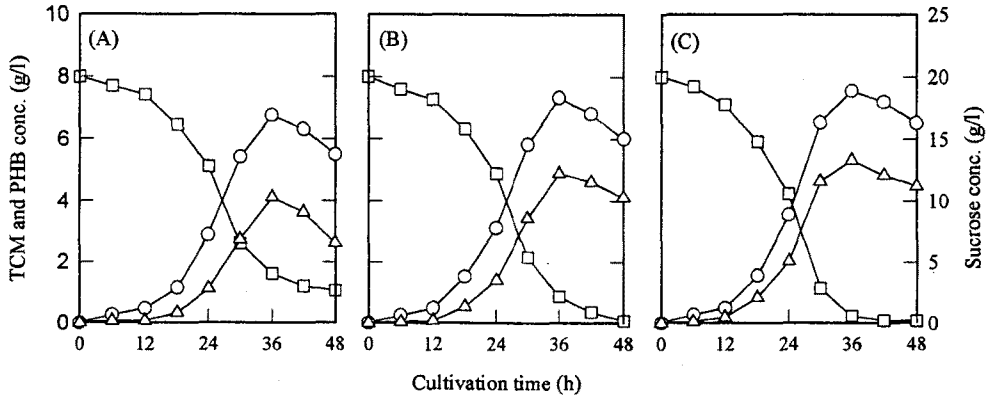

Fig. 4. Effect of supplements of citrate and tyrosine on cell growth and PHB accumulation of *A. latus*.
Cultivation: Minimal medium (per liter), 20 g of sucrose, 60 mg of citrate, 60 mg of tyrosine, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 8 h. (A) No supplement, (B) citrate supplement, (C) tyrosine supplement. ○, total cell mass (TCM); △, PHB concentration; □, sucrose concentration.

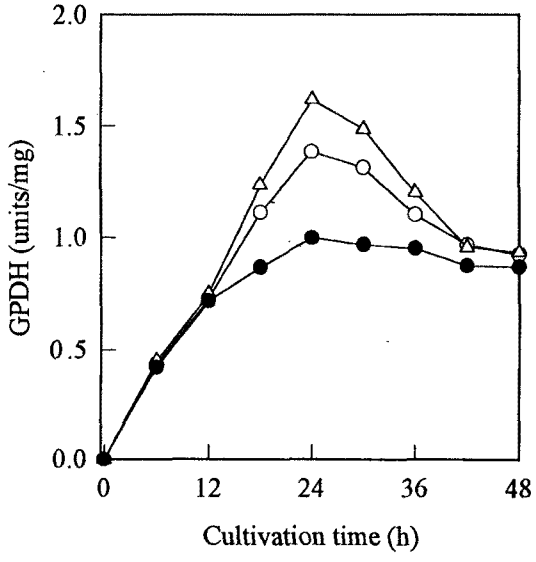

Fig. 5. Effect of the supplements of citrate and tyrosine on the specific activity of glucose-6-phosphate dehydrogenase (GPDH) of *A. latus*.
Cultivation: Minimal medium (per liter), 20 g of sucrose, 60 mg of citrate, 60 mg of tyrosine, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 48 h. ●, no supplement; ○, citrate supplement; ▲, tyrosine supplement.

Enzymatic Activities of *A. latus* during Cultivation

Glucose-6-phosphate dehydrogenase. Fig. 5 shows the effect of supplements of metabolic regulators, citrate and tyrosine, during cultivation of *A. latus* in the minimal medium on the specific activity of glucose-6-phosphate dehydrogenase, which is closely related to PHB biosynthesis by generating NADPH used as reducing power for the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA. The specific activity of glucose-6-phosphate dehydrogenase increased after supplements of citrate or tyrosine to around 1.23 and 1.46 times higher than the control, respectively, and tyrosine showed a much more significant effect compared to citrate.

The variation of the specific activity of glucose-6-phosphate dehydrogenase by supplement of metabolic regulators accorded well with the increased sucrose consumption rates during cultivation, and also with the previous observation (13) where tyrosine showed a better result for sucrose utilization. This indicates that the activation of glucose-6-phosphate, the key regulatory enzyme of sucrose catabolism, is essential for the effective biosynthesis of PHB in *A. latus*.

PHB biosynthetic enzymes. The effect of metabolic regulators, citrate and tyrosine, on the specific activities of PHB biosynthetic enzymes, β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, are depicted in Fig. 6A and 6B. Citrate supplement did not significantly influence the activity of PHB synthase, meanwhile, the activity of β-ketothiolase and acetoacetyl-CoA reductase was increased by around 1.20 and 1.30 times by citrate, respectively. Meanwhile, the activation of β-ketothiolase can be explained by the inductive effect of a high level of acetyl-CoA that accumulated as a result of the depression of citrate synthetase by citrate through product inhibition. The acetyl-CoA is known to activate β-ketothiolase and functioned as an activator for PHB biosynthesis (16).

Tyrosine influenced the activities of β-ketothiolase and acetoacetyl-CoA reductase more noticeably compared to citrate. The activation of the above two enzymes may be due to the increased availability of acetyl-CoA and NADPH because sucrose can be effectively utilized by the activation of the key regulatory enzyme of sucrose catabolism, glucose-6-phosphate dehydrogenase, with the supplement of tyrosine.

PHB degradation enzymes. Fig. 7A and 7B illustrate the effect of metabolic regulators on the variations of ac-

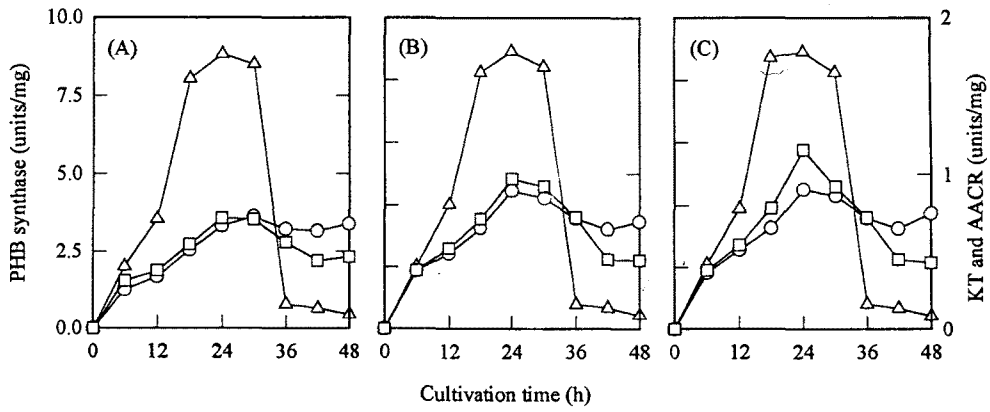

Fig. 6. Effect of the supplments of citrate and tyrosine on the specific activities of β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase of *A. latus*.

Cultivation: Minimal medium (per liter), 20 g of sucrose, 60 mg of citrate, 60 mg of tyrosine, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 48 h. (A) No supplement, (B) citrate supplement, (C) tyrosine supplement. ○, β-ketothiolase (KT); □, acetoacetyl-CoA reductase (AACR); △, PHB synthase (PHBS).

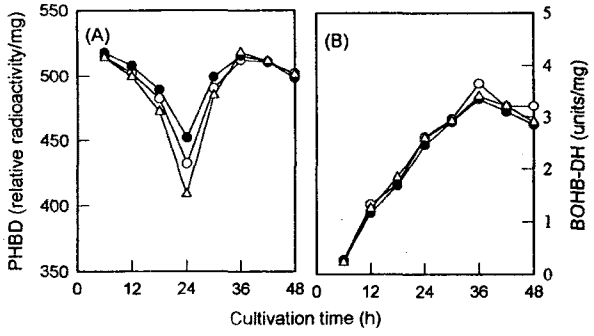

Fig. 7. Effect of the supplements of citrate and tyrosine on the specific activities of PHB depolymerase (PHBD) (A) and β-hydroxybutyrate dehydrogenase (BOHB-DH) (B) of *A. latus*.

Cultivation: Minimal medium (per liter), 20 g of sucrose, 60 mg of citrate, 60 mg of tyrosine, 2 g of $(NH_4)_2SO_4$, pH 7.0, 30°C, and for 48 h. ●, no supplement; ○, citrate supplement; △, tyrosine supplement.

tivity of PHB degradation enzymes, PHB depolymerase and β-hydroxybutyrate dehydrogenase. The variation patterns of PHB depolymerase for both citrate and tyrosine were not much different with that of the control, however, activities showed a lower level compared to that of the control. PHB depolymerase was decreased more severely by tyrosine, corresponding to 452.120 relative activity/mg protein. This severe effect of tyrosine can be explained by the previous observation in which PHB depolymerase may be controlled by the PTS system of sucrose transport and its activity is influenced by tyrosine more significantly. Meanwhile, the PHB degradation enzyme, β-hydroxybutyrate dehydrogenase, was not affected by metabolic regulators. This could be because β-hydroxybutyrate dehydrogenase is not regulated by the PTS system.

The reason of the increased total cell mass and PHB accumulation by tyrosine can be attributed mainly to the enhanced mechanism of sucrose utilization through the increased glucose-6-phosphate dehydrogenase activity, which activates PHB biosynthetic enzymes through the accelerated accumulation of acetyl-CoA and represses PHB degradation enzymes through the activated sucrose transport system. The efficiency of PHB biosynthesis in *A. latus* could be improved through the modification of various enzyme systems related to the glycolysis, TCA cycle, and biosynthesis and degradation of PHB.

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