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Kinetic analysis on formation of poly(3-hydroxybutyrate) from acetic acid by *Ralstonia eutropha* under chemically defined conditions

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Batch cultures of *Ralstonia eutropha* in chemically defined media with acetic acid (HAc) as the sole carbon source were conducted to investigate acetate utilization, formation of poly(3-hydroxybutyrate) (PHB) and growth of active biomass (ABM) under different carbon to nitrogen (C/N) weight ratios. The specific acetate utilization rate based on ABM approached 0.16 g/g ABM h^{-1}, which was not affected very much by the extracellular HAc concentration from 1 to 5 g/l, but was affected by the C/N weight ratio. A low C/N ratio or high nitrogen supply sped up the specific acetate utilization rate to produce more ABM and less PHB. A high HAc concentration (>6 g/l), however, depressed acetate utilization as well as the ABM growth and PHB formation. A high cell mass concentration enhanced the tolerance of *R. eutropha* to the toxicity of HAc at pH 7 to 8.5. The viscosity-average molecular size of PHB generally increased first and then declined in batch cultures. Larger PHB molecules and less PHB per ABM were produced at a low C/N ratio with enough nutrient nitrogen than those under a high C/N ratio with less nutrient nitrogen available. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 121–126.

Keywords: polyhydroxybutyrate; PHB; biodegradable thermoplastics; acetic acid; *Ralstonia eutropha*; *Alcaligenes eutrophus*

Introduction

Poly(3-hydroxybutyrate) (PHB) is a representative member of a family of polyesters, polyhydroxyalkanoates (PHAs) formed by many bacterial species as carbon and energy reserves under unbalanced growth conditions including nitrogen limitation [1]. As a biodegradable polymer, PHB has similar mechanical properties to those of commodity thermoplastics such as polypropylene and may be an alternative to the nonbiodegradable plastics [6]. PHB also plays a key role in wastewater treatment for enhanced biological phosphorus removal with polyphosphate (poly-P) being accumulated in bacterial cells at the expense of PHB degradation [10].

Ralstonia eutropha is an extensively studied bacterium of both basic and applied research on formation of PHAs. This facultative chemoautotrophic species can accumulate PHAs up to 80% of the dry cell mass (DCM) by utilizing various carbon sources including carbohydrates, organic acids and alcohols [1]. Homopolymer PHB is usually formed when the bacterium is fed organic acids of even carbon numbers such as acetic and butyric acids. In *R. eutropha*, PHB is synthesized from acetyl-CoA by a sequence of three reactions catalyzed by 3-ketothiolase (acetyl-CoA acetyltransferase; EC 2.3.1.9), NADPH-dependent acetoacetyl CoA reductase (hydroxybutyryl CoA dehydrogenase; EC 1.1.1.36) and PHB synthase [6]. With PHA synthesis genes expressed in *Escherichia coli*, different PHB formation from glucose as a sole carbon source was observed [7]. The strains with high PHB content (85% DCM) did not release acetate into the broth, but strains with low PHB content (20% DCM) released a large amount of acetate to the broth. Utilization of acetic acid (HAc) seems an essential step in PHB formation. HAc is also a major fermentative acid from anaerobic digestion of organic wastes from which PHA can be produced in a two-stage bioprocess [4,5]. Compared to studies on PHA formation from other organic acids such as propionic and butyric acids [2,12], few kinetic studies have been conducted on formation of PHB from HAc including substrate utilization and cell reproduction on acetate [8,11].

In this work, we investigated the uptake rate of HAc by active biomasses (ABMs) of *R. eutropha* in chemically defined media and the rates of PHB formation and ABM growth on HAc. Using kinetic analysis, we studied the effects of carbon to nitrogen (C/N) weight ratio on the yields of ABM and PHB from HAc as well as the influence of extracellular acetate concentration on the acid utilization rate. The effects of medium C/N ratio on PHB molecular size and the amount of PHB formed per ABM are also reported.

Materials and methods

R. eutropha (*Alcaligenes eutrophus* ATCC 17699) was maintained on agar slants (grams per liter); 10 fructose, 10 yeast extract, 10 peptone, 10 beef extract and 15 agar. The bacterium was cultivated in a nutrient-rich medium containing (grams per liter) 5 yeast extract, 5 peptone, 2.5 $(NH_4)_2SO_4$ and 2.5 beef extract agitated at 180 rpm, at 30°C for 24 h. The cell mass was harvested by centrifugation at 10,000×*g* for 10 min, washed with 0.8% NaCl solution and resuspended at a predetermined turbidity in a mineral solution containing (per liter) 3.8 g Na_2HPO_4, 2.65 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 1 ml trace element solution. The trace solution contained (mg/l): 200 $Fe(NH_4)SO_4$, 5 $ZnSO_4 \cdot 7H_2O$, 5 $MnCl_2 \cdot 4H_2O$, 2 $CuSO_4 \cdot 5H_2O$, 2 $NaB_4O_2 \cdot 10H_2O$ and 2 $NaMoO_4 \cdot 2H_2O$. Sodium acetate and NH_4Cl were added according to predetermined concentrations and (C/N) ratios in two ways: a

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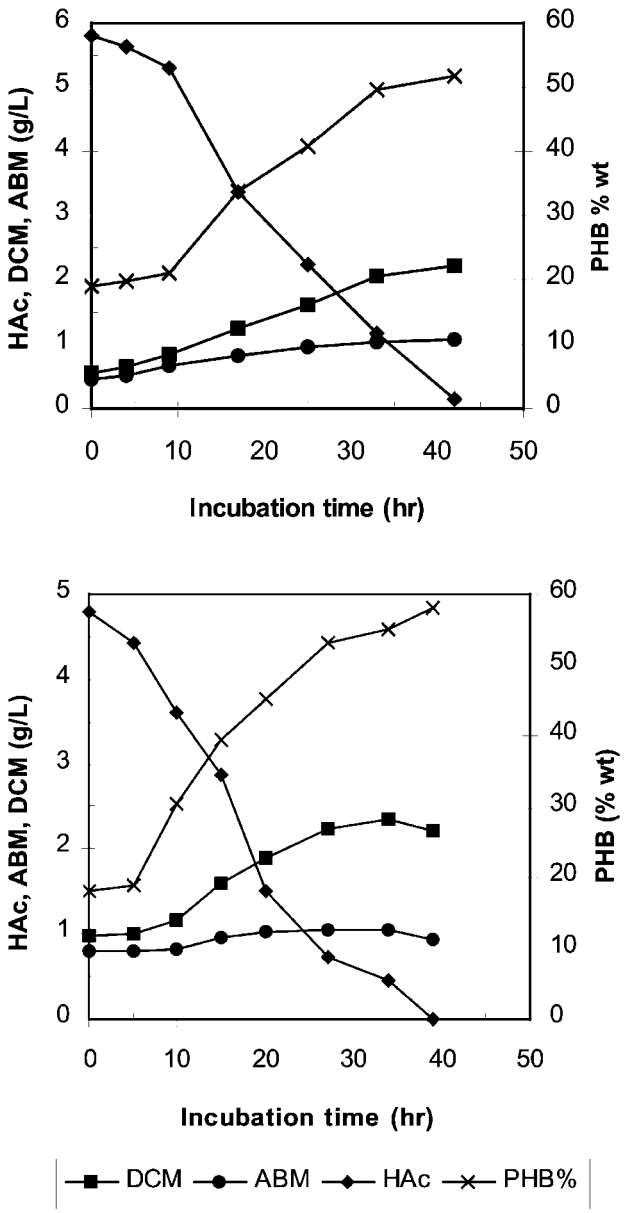

Figure 1 Time courses of DCM, ABM, HAc and PHB content in two cultures with initial C/N weight ratios of 30 (top) and 76 (bottom), respectively.

fixed initial acetate concentration (5–6 g/l) with different initial NH_4Cl concentrations (0.1–0.3 g/l), and a constant initial NH_4Cl (0.3 g/l) concentration with various initial acetate concentrations (1–9 g/l). The former was to study the effect of nutrient nitrogen on acetate utilization, cell growth and PHB formation and the latter to study the effect of HAc concentration. The cultures were conducted in flasks agitated at 180 rpm and at 30°C and sampled for determination of HAc, DCM, ABM and PHB. The medium pH after inoculation was around 7.1 and rose with time to 8.5 at the end of batch cultivation.

The total HAc concentration in the medium was determined by gas chromatography (Hewlett Packard 5890II) equipped with a Nukol fused silica capillary column (0.25 mm×30 m, Supelco). The sample was acidified to pH 2 with a phosphoric acid solution and filtered through a 0.2-μm pore size cellulose nitrate membrane. HAc was eluted with helium at 10 ml/min using a temperature program from 120 to 190°C at 10°C/min. Although HAc was mainly in its anionic form (>99.5%) at the medium pH (7–8.5), we report the total amount of acetate as free HAc for easy comparison with literature data. ABM was measured for monitoring cell growth and defined as the residual cell mass after PHB was extracted from the cell mass (DCM). They were determined as follows. The cell mass of a broth sample was harvested by centrifugation at 10,000×g for 10 min, washed with distilled water and freeze-dried. Approximately 0.1 g DCM was dispersed in 10 ml chloroform, sealed in a glass tube, and kept at 60°C for 24 h in a dry bath. More than 97% PHB was extracted from the DCM under these conditions. After it had cooled to room temperature, the chloroform solution was filtered through a glassfiber filter. The weights of PHB and residual cell debris (or ABM) were measured after the solvent was completely vaporized. The measurement error was less than 5%. The amount of nonpolymer materials such as lipids extracted into chloroform was less than 4 wt.% of the polymer as determined by hexane precipitation of PHB from the chloroform solution. The molecular size of PHB was estimated based on viscosity measurement to give a viscosity-average molecular mass, M_v [3]. Predetermined amounts of PHB were dissolved in chloroform. The intrinsic viscosity (η) of PHB–chloroform solutions was determined at 30±0.1°C using a Ubbelohde viscometer, and M_v was calculated according to the Mark–Houwink–Sakurada equation: $\eta=K'(M_v)^a$, where $K'=7.7\times10^{-5}$ dl/g and $a=0.82$ for PHAs [9].

Results and discussion

Effect of C/N ratio on acetate utilization and yields

Figure 1 shows the time courses of HAc, ABM, DCM and PHB content in two batch cultures with initial C/N weight ratios of 30 and 76, respectively. The initial C/N ratio was controlled by adding different amounts of ammonium chloride but the same amount of sodium acetate. Although the two cultures had different lag times, more than 80% of the initial HAc (5—6 g/l) was utilized by *R. eutropha* in a linear mode of concentration versus time. The linear relationship between concentration and time was also observed in the formation of DCM, ABM and PHB. By correlating the data in the linear zone (from the 5th to the 33rd hour and from the 5th to the 26th hour in two batch cultures, respectively, Figure 1), we found volume-based utilization rate of HAc and formation rates of ABM and PHB listed in Table 1. The correlation coefficients (r^2)

Table 1 Volumetric utilization rate of HAc and formation rates of ABM and PHB

Species	Rate per culture volume (g/l h^{-1})	
	C/N=30 (wt)	C/N=76 (wt)
HAc	0.16 (0.98)	0.17 (0.98)
ABM	0.018 (0.97)	0.013 (0.87)
PHB	0.031 (0.97)	0.046 (0.99)

The batch cultures had initial C/N weight ratios of 30 and 76, respectively. The linear correlation coefficients (r^2) between the concentrations and time are given in parentheses.

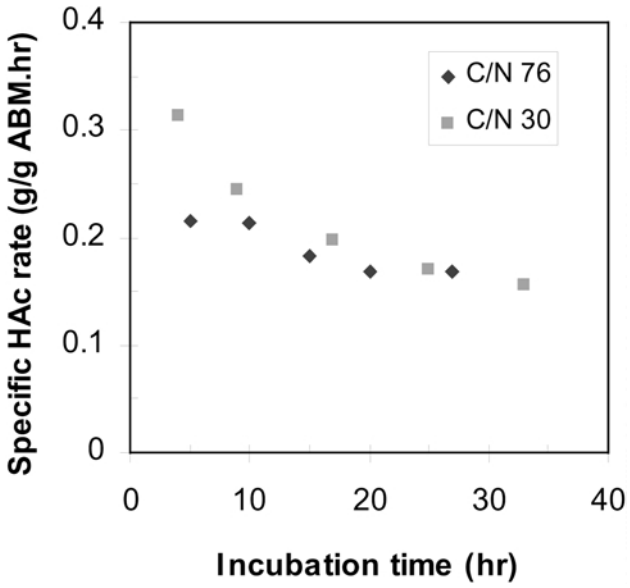

Figure 2 Change in the specific utilization rate of HAc with time in two batch cultures of two initial C/N weight ratios of 30 and 76, respectively. The specific rates are based on ABM.

ranging from 0.97 to 0.99 indicate the high linear correlations except the formation of ABM (r^2=0.87) under the high C/N ratio (C/N=76).

Because of the differences in the initial ABM and reproduction rate of ABM in the two cultures, the volume-based rates are further converted to specific rates per ABM. Figure 2 compares the change in specific acid utilization rate with incubation time at two initial C/N weight ratios. Under the high initial C/N ratio (C/N=76), the specific utilization rate of HAc declined slightly from 0.22 g/g ABM h^{-1} to a constant value around 0.17 g/g ABM h^{-1} regardless of a great change in extracellular HAc concentration from 4.5 to 0.8 g/l. In this concentration range, more than 80% of the initial amount of HAc was utilized in a linear mode giving a slope from which the specific utilization rate could be reliably estimated (Table 1). Below the level of 0.8 g/l, the linearity became worse (r^2<0.8), and hence the quality of the rate data. The small change in the specific rate implies that acetate uptake was not affected very much by the extracellular acetate concentration from 4.5 to 0.8 g/l. Under a relatively low initial C/N weight ratio (C/N 30), however, the specific rate declined from 0.32 to 0.16 g/g ABM h^{-1} corresponding to the decline of acetate concentration from 5.3 to 1 g/l, a similar concentration range as discussed above. Because the acetate utilization rate was not affected very much by medium acetate concentration, (see additional evidence below) the two groups of data under different C/N weight ratios show the effect of nutrient nitrogen on the specific acetate utilization rate. Under high C/N ratio or nitrogen limitation (C/N 76), HAc was utilized mainly for PHB formation and energy replenishment essential to polymer synthesis and cell activity maintenance. Acetate uptake, therefore, was controlled by secondary metabolism in the stationary phase with little ABM reproduction. It might not be controlled by passive diffusion of HAc molecules into the cells because passive molecule diffusion depends on a concentration gradient across the cell membrane and increases with extracellular concentration. Under a relatively low C/N weight ratio or less nitrogen limitation (C/N 30), at the beginning of batch culture in particular, a considerable amount of HAc was also consumed for biomass reproduction as the carbon and energy source. The synthesis of new cell mass involved primary metabolism of acetate and sped up its uptake rate. Figure 3 shows the specific formation rates of ABM and PHB against the specific utilization rate of HAc. The slopes of the straight lines give the yields of ABM and PHB over the consumption of HAc. Less ABM (Y_{ABM}=0.07) and move PHB (Y_{PHB}=0.27) were formed under high C/N ratio (C/N=76) than those (Y_{ABM}=0.11 and Y_{PHB}=0.19) under low C/N ration (C/N=30).

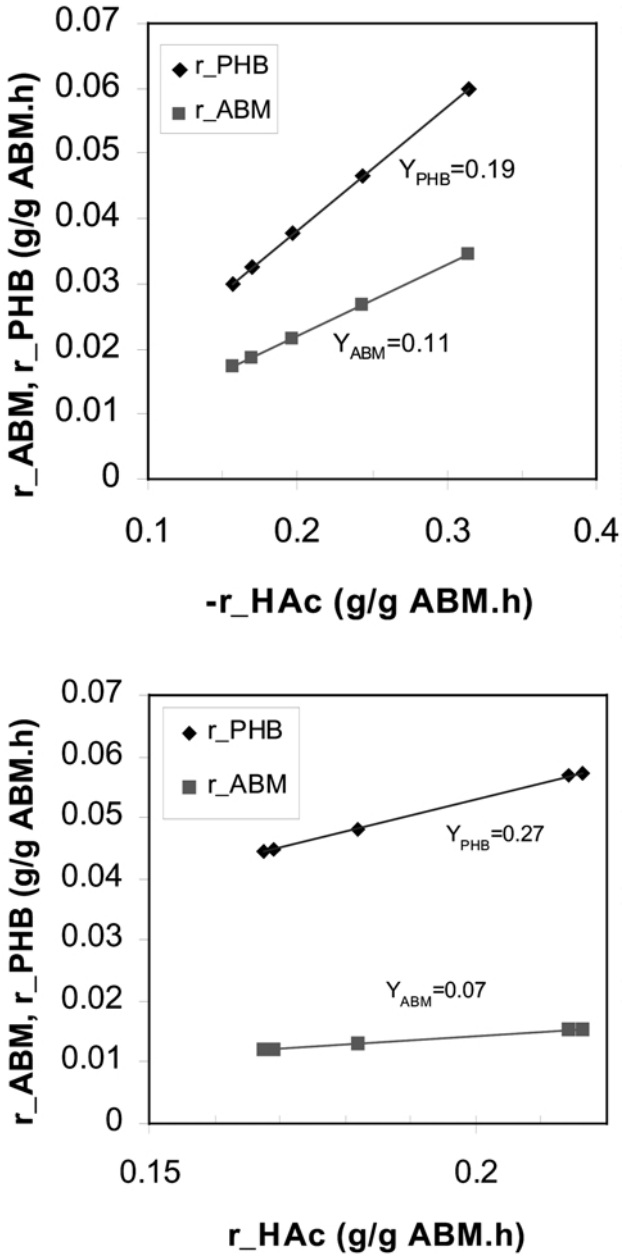

Figure 3 Specific formation rates of ABM and PHB versus the specific utilization rate of HAc with two inital C/N ratios: 30 (top) and 76 (bottom). The slopes of the two straight lines represent the yields of ABM (Y_{ABM}) and PHB (Y_{PHB}).

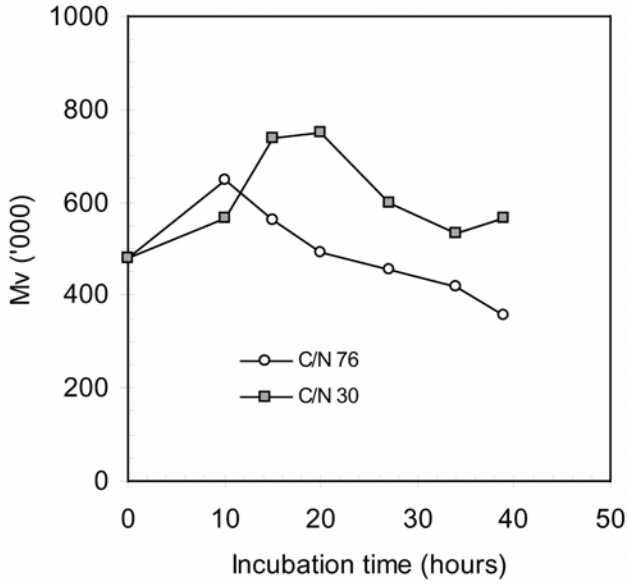

Figure 4 Time courses of viscosity-average molecular mass (M_v) of PHB formed under two initial C/N ratios, 30 and 76.

Effect of C/N ratio on polymer size

The viscosity-average molecule size (M_v) of PHB formed under two C/N weight ratios was monitored to give time courses as shown in Figure 4. The PHB size increased first and then declined under both C/N ratios. In general, the polymer size was bigger under low C/N ratio than under high C/N ratio. Cultivating the cells in chemical media with a wide range of C/N weight ratios from 4 to 72 revealed the phenomena shown in Figure 5. It should be emphasized that these molecular sizes were measured after 24 h cultivation and were not the largest ones under different C/N weight ratios as shown in Figure 4. With enough nitrogen, the cells synthesized large PHB molecules, but less PHB per ABM (PHB/ABM). By increasing the initial C/N ratio from 4 to 72, the molecular size was decreased by 58%, from 820,000 to 520,000, and the amount of PHB per ABM increased by 140%, from 0.5 to 1.2. More polymer synthesis centers might be triggered inside the cells under nitrogen-limited conditions than under nitrogen-rich conditions, which resulted in less monomer available for propagation of each growing polymer and a smaller PHB size at a constant uptake rate of HAc.

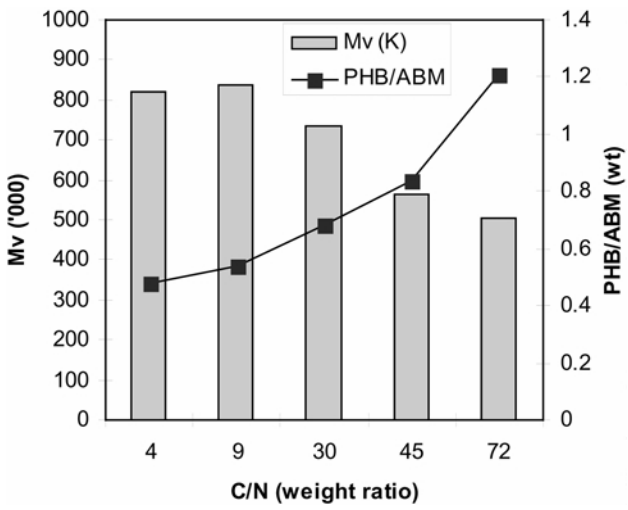

Figure 5 Effect of initial C/N ratio on PHB molecular size (M_v) and polymer/ABM ratio. The cells of *R. eutropha* grew on HAc (5 to 6 g/l) for 24 h.

Effect of HAc concentration on HAc utilization

Figure 6 shows the time courses of HAc, DCM, ABM and PHB content in two batch cultures with different initial C/N weight ratios. In contrast to the results in Figure 1 where the C/N weight ratio was controlled by changing the NH_4Cl concentration at a fixed acetate concentration, the cultures depicted in Figure 6 were prepared by changing acetate concentration at a constant NH_4Cl concentration (0.3 g/l). Similar to Figure 1, acetate concentration declined linearly with time to give constant volume-based acetate utilization rates, 0.17 g HAc/l h^{-1} (r^2=0.98) with initial C/N weight ratio 15, and 0.15 g HAc/l h^{-1} (r^2=0.98) with initial C/N

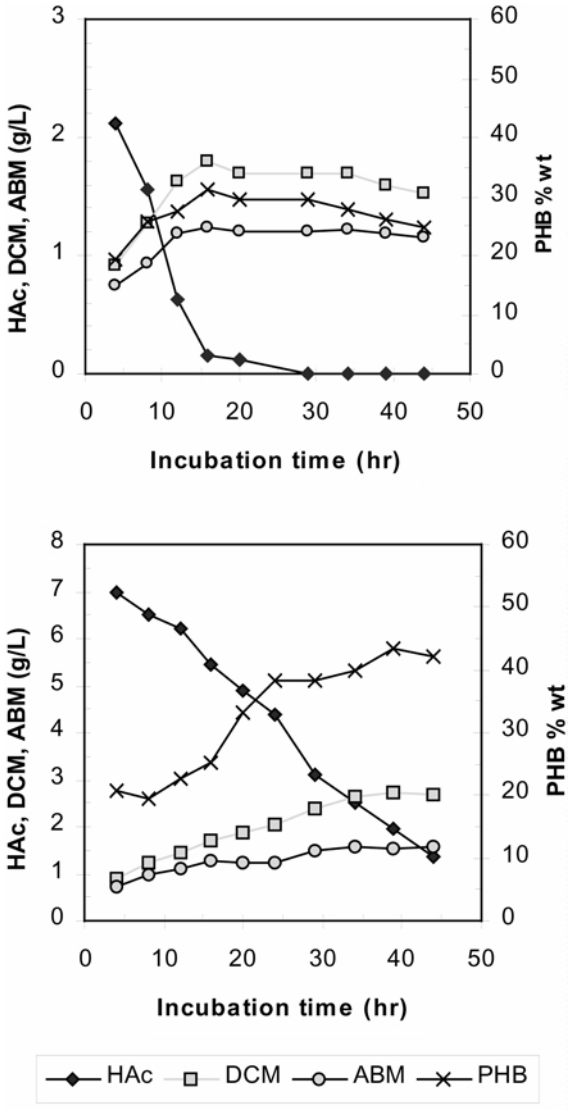

Figure 6 Time courses of HAc, DCM, ABM, and PHB content in batch cultures with initial NH_4Cl concentration of 0.3 g/l and initial HAc concentrations 3 g/l (top) and 9 g/l (bottom). The corresponding C/N ratios were 15 and 45, respectively.

of 45. The values are very close to those in Table 1. Considering the differences of ABM concentration and cell growth in the cultures, specific acetate utilization rates based on a unit mass of ABM were calculated and are compared in Figure 7. In order to observe a possible correlation between the acetate utilization rate and the medium acetate concentration, the specific rate is plotted against the HAc concentration in the broth. For the initial acetate concentrations of 3 and 6 g/l, respectively, the specific acetate utilization rate approached a constant value around 0.15 g/g ABM h^{-1} with acetate concentration declining from a high level at the beginning of batch culture to a low level. But the rate of decline was not closely associated with the change in acetate concentration in the two batches. No obvious relationship is evident between the acetate utilization rate and the medium concentration of HAc, which is in agreement with the results in Figure 2. As discussed before, the C/N weight ratio affects acetate metabolism among PHB, ABM and energy supply. Reproduction of ABM increases the acetate uptake and specific utilization rate whereas formation of PHB with necessary energy replenishment leads to a constant utilization rate of HAc. Based on a Monod type model for cell growth or Michaelis–Menten type model for enzymatic polymer synthesis, it is reasonable to assume that the specific acetate utilization rate is a 0th-order reaction with respect to acetate concentration or that the intracellular acetate has a high affinity for the enzymes.

Inhibitory effect of HAc and cell tolerance

Figure 7 also shows a low specific acetate utilization rate with an initial HAc concentration of 9 g/l. It might be attributed to a depression or toxic effect of a high acetate concentration in *R. eutropha*. Figure 8 shows the growth of ABM and PHB synthesis at initial acetate concentrations from 1 to 9 g/l. The medium had the same initial NH_4Cl concentration but two inocula (0.25 vs. 0.45 g DCM/l). With an acetate concentration below 5 g/l, increase in acetate concentration led to production of more ABM and also more PHB. Further raising the initial acetate concentration caused a depression of formation of both ABM and PHB. This inhibitory effect was much more significant at a low seed concentration (DCM=0.25 g/l) than at a high seed concentration (DCM=0.45 g/l), which implies that a high cell mass concentration can, to some extent, enhance the tolerance of *R. eutropha* to the toxicity of HAc.

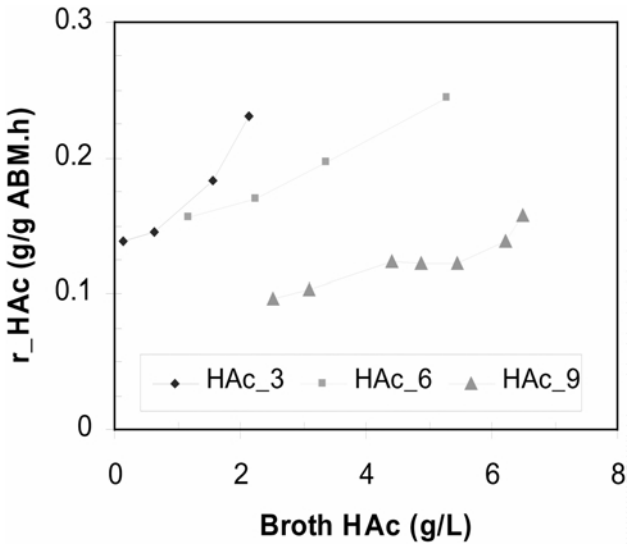

Figure 7 Specific utilization rate of HAc versus extracellular HAc concentration in batch cultures started with three initial HAc concentrations, 3, 6 and 9 g HAc/l, at an initial NH_4Cl concentration of 0.3 g/l. Their corresponding inital C/N ratios were 13, 30 and 45.

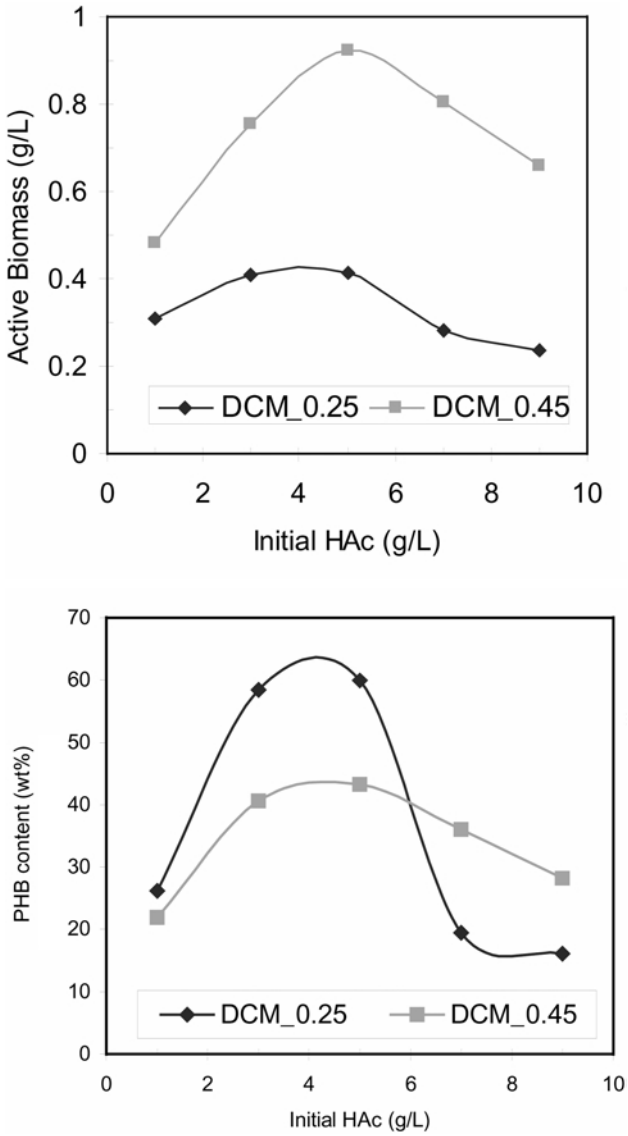

Figure 8 Toxic effect of high HAc concentration on ABM reproduction (top) and PHB formation (bottom). Initial DCM concentration in media was controlled at 0.25 and 0.45 g/l, respectively. The cultures were incubated for 24 h.

Conclusion

In a chemically defined medium, *R. eutropha* took up HAc for reproduction of ABM, synthesis of PHB and production of energy. The uptake of extracellular HAc was not controlled by passive molecule diffusion of acetate across the cell membrane, but by the consumption rate of intracellular HAc. The specific acetate utilization rate based on ABM approached 0.16 g/g ABM h^{-1}, and was affected not very much by the extracellular HAc concentration, but by the C/N weight ratio. A low C/N ratio or nutrient nitrogen-rich medium led to increased ABM reproduction

and acetate utilization but to decreased PHB formation. A high HAc concentration (>6 g/l), however, can have an inhibitory effect on cell growth, PHB formation and acetate utilization. Using a high cell mass concentration can, to some extent, relieve the inhibitory effect. The medium C/N weight ratio also has an effect on PHB molecular size PHB formed per ABM. Less PHB and larger polymer molecules are formed under nutrient-rich conditions than under nutrient-limitation conditions.

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