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ORIGINAL PAPER

Characterization of new isolated *Ralstonia eutropha* strain A-04 and kinetic study of biodegradable copolyester poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) production

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Abstract A new isolated bacterial strain A-04 capable of producing high content of polyhydroxyalkanoates (PHAs) was morphologically and taxonomically identified based on biochemical tests and 16S rRNA gene analysis. The isolate is a member of the genus *Ralstonia* and close to *Ralstonia eutropha*. Hence, this study has led to the finding of a new and unexplored *R. eutropha* strain A-04 capable of producing PHAs with reasonable yield. The kinetic study of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] production by the *R. eutropha* strain A-04 was examined using butyric acid and γ–hydroxybutyric acid as carbon sources. Effects of substrate ratio and mole ratio of carbon to nitrogen (C/N) on kinetic parameters were investigated in shake flask fed-batch cultivation. When C/N was 200, that is, nitrogen deficient condition, the specific production rate of 3-hydroxybutyrate (3HB) showed the highest value, whereas when C/N was in the range between 4 and 20, the maximum specific production rate of 4-hydroxybutyrate (4HB) was obtained. Thus, the synthesis of 3HB was growth-limited production under nitrogen-deficient condition, whereas the synthesis of 4HB was growth-associated production under nitrogen-sufficient condition. The mole fraction of 4HB units increased proportionally as the ratio of γ–hydroxybutyric acid in the feed medium increased at any value of C/N ratio. Based on these kinetic studies, a simple strategy to improve P(3HB-*co*-4HB) production in shake flask fed-batch cultivation was investigated using C/N and substrate feeding ratio as manipulating variable, and was successfully proved by the experiments.

Keywords P(3HB-*co*-4HB) · *Ralstonia eutropha* · 16S rRNA gene analysis · Kinetic study

Introduction

A majority of disposed plastic materials cause extensive solid waste pollution problem and hazardous ecosystem. Among various waste management techniques, incineration is a waste disposal method involving the combustion of plastic waste at high temperatures. During the combustion of plastic waste, carbon dioxide is released into the atmosphere, leading to global warming and continuously causing variation in the high level of temperatures in many countries. Moreover, the influence of the world crude oil market led to fears that the price of fossil fuels can no longer be regarded as reliable, and set the stage for the searching of alternative types of plastic materials [2]. Therefore, the next generation of polymers goes beyond more uses of biodegradable polymer in conventional applications. They are biodegradable, meaning that

The nucleotide sequence 1,378 bp reported in this study will appear in the GenBank nucleotide sequence database under accession number EF988626.

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the materials can be degraded by microbiological process and the degrading products are not harmful to the environment. Biodegradable polymers are already being used in automobile interiors and in consumer electronics [5]. This means that the use of biodegradable polymers is considered as an effective method of reducing pollution problem and is likely to continue to expand in various applications.

Polyhydoxyalkanoates (PHAs) have attracted commercial and academic interest because they are renewable resource-based plastics having biodegradable and biocompatible properties that offer a wide range of potential applications. A growing number of other uses for these materials include medical and engineering materials [16]. To date, PHAs are aimed at medical devices and barrier packaging in addition to standard packaging and disposable uses [13]. The physical and mechanical properties of PHAs can be manipulated to provide a broad range of applications, in some cases matching the performance of engineered thermoplastics.

It has been reported that P(3HB-*co*-4HB)s have been tried in applications in soft tissue engineering due to their unique mechanical properties [8]. Recently, P(3HB-*co*-4HB) has been found to be useful in biomedical applications [14]. In addition, the homopolymer of P(4HB) has already been used for tissue engineered heart valve scaffold and viable ovine blood vessels [4]. Thus, the prospects of producing P(3HB-*co*-4HB) with different monomer units would widen the potential usefulness, and P(3HB-*co*-4HB) will become one of the suitable candidates for commodity materials. Nevertheless, there has been a few efforts on the kinetic study of P(3HB-*co*-4HB) production.

This study reports the taxonomical identification of a new PHA producing *Ralstonia eutropha* strain A-04 based on biochemical tests and 16S rRNA gene analysis. The authors show that the newly isolated *R. eutropha* strain A-04 can synthesize P(3HB-*co*-4HB) efficiently in shake flask fed-batch cultivation. The effects of changing the ratios of C/N and carbon sources on kinetics of P(3HB-*co*-4HB) production were investigated, and a simple strategy to enhance PHAs production was investigated and verified.

Materials and methods

Microorganism

The bacterium strain A-04 was isolated from soil in Thailand. The strain was maintained by monthly subculture on nutrient agar slants. Stock cultures were stored at −80 °C in a 20% (v/v) glycerol solution.

Morphological and taxonomical identification

The cell morphology and PHB granules were observed by transmission electron microscope (TEM). For TEM, the diluted culture from the production medium was fixed in 2% (v/v) glutaraldehyde containing 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and was postfixed in 1% (w/v) osmium tetroxide. Cells were dehydrated in ascending series of ethanol from 35 to 100%, and were embedded in Spurr resin (EMS, PA, USA). Thin sections were prepared with the LKB 2088 Ultrotome V (Surrley, U.K.), stained with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate, and were examined with the JEOL (TEM 2100) transmission electron microscope at an accelerating voltage of 80 kV. Cellular morphology after Gram staining was also checked by light microscopy. The Hucker method [15] was used for Gram staining. The catalase activity assay was performed by the detection of bubble formation in 3% (w/v) hydrogen peroxide solution after incubating cells in the nutrient medium for 18–24 h. The ability to grow utilizing the substrates such as glucose, fructose, lactose, sucrose, maltose, and galactose was tested by inoculating the strain A04 in the basal medium supplemented with 2% (w/v) of each carbohydrate. The cells were incubated at 30 °C for 3 days. Starch hydrolysis, gelatin hydrolysis, hydrogen sulfide production, nitrate reduction, Methyl Red-Voges Proskauer (MR-VP) test, and citrate utilization test were examined. Acid production from carbohydrates was determined in the basal medium supplemented with various carbohydrates [20]. All the assays were performed thrice.

The 16S rRNA gene sequencing

Genomic DNA was prepared from colonies, using the Wizard Genomic DNA Purification Kit (Promega, USA) and used for PCR. The 16S rDNA was amplified using the primer 518F for forward primer (5′-CCAGCAGCCG CGGTAATACG-3′) and 800R for reverse primer (5′-T ACCAGGGTATCTAATCC-3′). The PCR reaction and 16S rDNA sequencing was performed at Macrogen service center (Macrogen Inc., Seoul, Korea). To avoid misreading owing to PCR error, sequencing of the PCR fragment was repeated at least twice. The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Besthesda, MD) was used for gene homology search with the standard program default. The nucleotide sequences of the 16S rRNA genes from strain A04 has been deposited in the EMBL/GenBank/DDBJ databases (GenBank accession no. EF988626).

Phylogenetic analysis of 16S rRNA gene sequence

The nucleotide sequences obtained were analyzed with DNASIS-MAC software (version 2.05; Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Multiple alignments of the determined sequences were performed using the CLUSTAL X program version 1.83 [21]. A phylogenetic tree was constructed by the neighbor-joining method [11], and phylogenetic analysis was performed using the PHYLIP version 3.572c package [7]. The percentage confidence was estimated by bootstrap analysis with 100 replications.

Inoculums preparation and media

The preculture medium consisted of 10 g/l yeast extract, 10 g/l polypeptone, 5 g/l beef extract, and 5 g/l $(NH_4)_2SO_4$. The preculture cultivation was performed in 250 ml shake flasks containing 50 ml of preculture medium and incubated in a rotary shaker at 200 rpm at 30 °C for 16 h. The cells were harvested by centrifugation and washed in order to remove residual nitrogen. The cells were resuspended and inoculated to the production medium. The production medium was a mineral salt medium consisting of 5.8 g/l K_2HPO_4, 3.7 g/l KH_2PO_4, 0.12 g/l $MgSO_4{\cdot}7H_2O$, 5 g/l sodium citrate, and 1 ml of trace element solution [1.67 g/l $CaCl_2{\cdot}2\ H_2O$, 2.78 g/l $ZnSO_4.7\ H_2O$, 0.29 g/l $FeSO_4.7\ H_2O$, 1.98 g/l $MnCl_2.4\ H_2O$, 0.17 g/l $CuCl_2.2H_2O$].

Precursor-substrates for PHAs and nitrogen source

Butyric acid, valeric acid, fructose, γ-hydroxybutyric acid, γ-hydroxybutyric acid lactone, and 1,4-butanediol were purchased from Sigma (Sigma, St. Louis, Mo) and used as carbon sources. Ammonium sulfate was used as nitrogen source. The amount of Ammonium sulfate was calculated based on mole ratio of carbon to nitrogen.

Intermittent fed-batch cultivation

The intermittent fed-batch cultivations were performed in 500 ml flasks containing 100 ml of the production medium. The cultivation was performed on the rotary shaker at 200 rpm at 30 °C for up to 96 h. The effect of the mole ratio of C/N on the relationship between the specific growth rate and the specific production rate was investigated for C/N of 4, 20, 40, 80, 200 and without nitrogen source in detail, where the ratio of γ-hydroxybutyric acid to total carbon sources was varied from 0, 25, 50, 75, 95, and 100 wt%, respectively. Carbon and nitrogen sources were added at 12 h interval so that the C/N was maintained at an appropriate value throughout the cultivation.

Analytical methods

Total cell mass was measured as dry-cell weight by filtering of culture broth using preweighted membrane filters of cellulose nitrate (pore size 0.45 μm, Sartorius, Goettingen, Germany) and drying at 80 °C for 2 days. The net biomass was evaluated as residual biomass concentration, which was calculated by subtracting the amount of PHAs from the total cell mass. PHAs in dried cells were methyl esterified in the mixture (1:1 v/v) of chloroform and methanol-sulfuric acid as described by Braunegg et al. [1]. The resulting methyl esters of monomers were quantified using gas chromatography (Model CP3800, Varian, USA) attached with cabowax-PEG capillary column (0.25 μm df, 0.25 mm ID, 60 m length, Varian, USA). Internal standard was benzoic acid and external standards were PHB and γ-hydroxybutyric acid. Concentrations of butyric acid, γ-hydroxybutyric acid, and 1,4-butanediol were also measured offline by gas chromatography (Model CP3800, Varian, USA) attached with CP-WAX 52CB capillary column (0.25 μm df, 0.25 mm ID, 30 m length, Varian, USA) and ethanol was used as an internal standard. The PHA compositions and its mole fraction were confirmed by ^{1}H NMR (Varian Inova 600 MHz; Palo Alto, CA, USA).

Results and discussion

Morphological and taxonomical identification

The physiological and biochemical characteristics of strain A-04 were examined and the results are shown in Table 1. The colonies on the nutrient agar plates were circular, low convex with entire margins, opaque, and moist. The bacterium was found to be a gram negative, short rod shape, nonspore-forming, and motile. It grew aerobically. It was capable of growth at 30 and 37 °C. It could not hydrolyze gelatin and starch. It was positive in the tests of catalase and citrate utilization. Hydrogen sulfide formation and MR-VP test were negative. Acid was produced weakly from glucose, fructose, lactose, sucrose, maltose and galactose. No gas was produced from all the tested carbohydrates. It could reduce nitrate. The strain A-04 could use a wide variety of carbohydrates as carbon sources for growth. Suitable nitrogen sources were yeast extract, peptone, tryptone, beef extract, and ammonium sulfate. The strain A-04 has an optimum growth temperature of 30 °C at which the specific growth rate is 0.2575 h^{-1} in a shaking flask containing preculture medium.

To identify this strain, the 16S rRNA genes were amplified by PCR using genomic DNA of this strain as a template. Nucleotide sequences of the DNA fragment

Table 1 Characterization of the isolated PHA producing strain A-04 based on biochemical test

Biochemical test	Result
Gram stain	Negative
Rod shape	+
Oxygen requirements	Aerobic
Endospore	−
Motility	+
Catalase	+
Growth at 37 °C	+
Growth at 2% (w/v) of NaCl	+
Hydrolysis of Gelatin	−
Hydrolysis of Starch	−
H_2S formation	−
Nitrate reduction	+
Methyl red Voges-Proskauer test	−
Citrate utilization	+
Acid produced from:	
Glucose	+/weak
Fructose	+/weak
Lactose	+/weak
Moltose	+/weak
Galactose	+/weak
Sucrose	+/weak

encoding of the 16S rRNA gene clearly demonstrated that the closet matches for the strain A-04 belong to genus *R. eutropha* (formerly *Alcaligenes eutropha*) species, with the highest identity to *R. eutropha* strain LMG 1199 (M32021) and *R. eutropha* strain H16 (AM260479), which were 99.41 and 99.78%, respectively. The data set used for the construction of the phylogenetic tree contained 1,348 nucleotides. A phylogenetic tree generated by the neighbor-joining method is shown in Fig. 1. The neighbour-joining analysis of 16S rRNA genes was rooted by referring to Gammaproteobacteria (*Pseudomonas aeruginosa*) and compared among Betaproteobacteria (*Comamonas* sp., *Burkholderia* sp., and *Ralstonia* sp.) with bootstrap support value of 100%. Hence, the strain A-04 was identified as *R. eutropha* and was named *R. eutropha* strain A-04. In addition, elemental analysis of dried-cell was made by Center for Organic Elemental Microanalysis, Kyoto University. The results showed that the element compositions of biomass for *R. eutropha* strain H16 are C 44.5%; H 6.6%; N 12.86%; O 29.69% with ash content of 5.31 (w/w%) and calculated mole ratio of C/N for biomass is 4.04 whereas those for *R. eutropha* strain A-04 are C 50.5%; H 7%; N 9.23%; O 30.44% with ash content of 2.86 (w/w%) and calculated mole ratio of C/N for biomass is 6.38.

Biosynthesis of PHAs from various carbon sources

The newly characterized *R. eutropha* strain A-04 was preliminarily investigated for the accumulation of PHAs using fructose, butyric acid, valeric acid, γ-hydroxybutyric acid, γ-hydroxybutyric acid lactone, and 1,4-butanediol as carbon sources. In this study, the comparison with polymer content and monomer composition of produced PHAs between *R. eutropha* strain A-04 and *R. eutropha* strain H16 was shown in Table 2. It was initially grown using 20 g/l fructose as a sole carbon in the production medium for 72 h in shake flasks, and the PHA produced was isolated and quantified at various time intervals. The highest PHB content of 78 wt% was produced at 60 h grater than

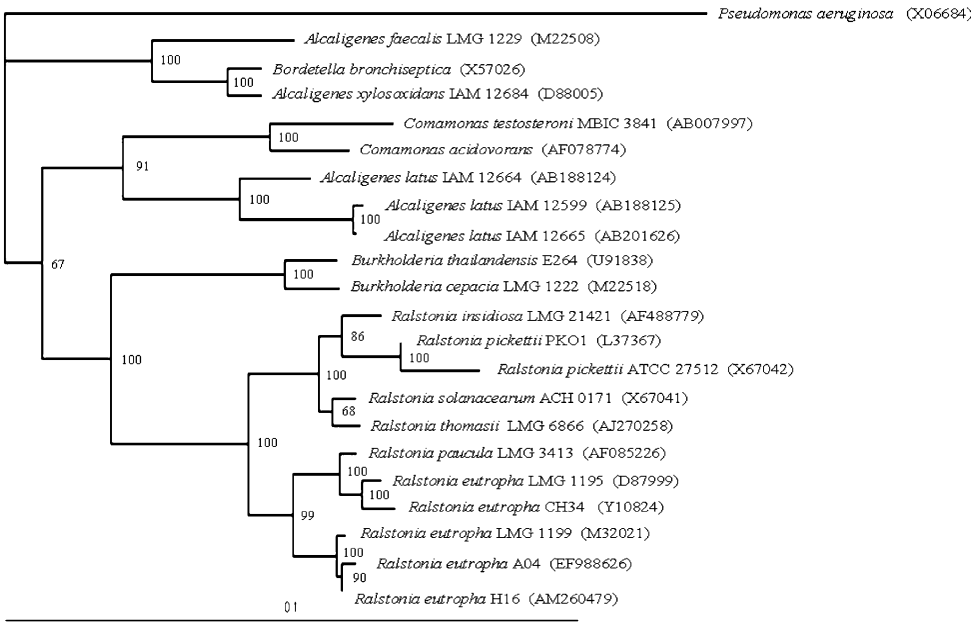

Fig. 1 Phylogenetic tree constructed based on 1,348 bp long fragment of the 16S rDNA sequences of 22 bacteria. The tree was constructed by neighbor-joining method and was rooted by referring to *Pseudomonas aeruginosa*. The number of branches refers to the percentage confidence estimated by bootstrap analysis with 100 replications. Analysis was performed by including 16S rRNA gene sequences deposited in GenBank (accession numbers are *indicated in parentheses*). Bar = 0.1 estimated substitution per sequence position

Table 2 Comparison with PHA content and monomer composition of PHAs produced by *R. eutropha* strain A-04 and *R. eutropha* H16 when grown on various carbon sources

Bacterium strain and carbon source	Biomass (g/l)	PHA content (wt%)	PHA composition (mol%)		
			3HB	3HV	4HB
R. eutropha strain A-04					
Fructose	6.8	78	100	0	0
Butyric acid	4.32	52	100	0	0
Valeric acid	3.14	20	21	79	0
γ-Hydroxybutyric acid	5.63	22	48	0	52
γ-Hydroxybutyric acid lactone	2.03	47	0	0	20
1,4-butanediol	4.35	43	88	0	12
R. eutropha strain H16					
Fructose	5.5	45	100	0	0
Butyric acid	2.6	51	100	0	0
Valeric acid	6.5	52	42	60	0
γ-Hydroxybutyric acid	4.9	19	69	0	31
γ-Hydroxybutyric acid lactone	4.1	21	83	0	17
1,4-butanediol	5.4	22	89	0	11

Cells were cultivated in 100 ml of production medium containing 20 g/l carbon source and 0.5 g/l$(NH_4)_2SO_4$ for 72 h at 30 °C in shake flask batch cultivation

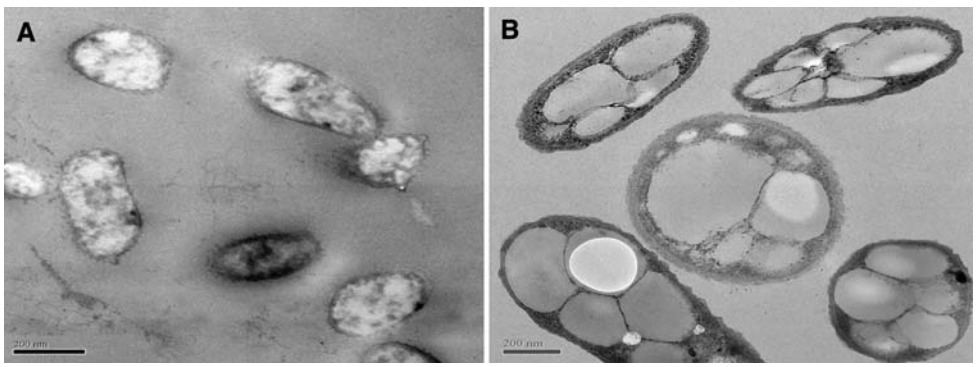

Fig. 2 Transmission electron micrograph of ultra-thin section of *R. eutropha* strain A-04 **a** typical short rod cells ranging from 0.6 to 1.0 μm, bar 1 μm. **b** *R. eutropha* strain A-04 containing PHB granules (*white fractions*). Bar 200 nm

that in *R. eutropha* strain H16. Typical cell characteristics of *R. eutropha* strain A-04 and cells containing PHB granules were examined by TEM, as shown in Fig. 2. As can be seen in Fig. 2a, cells were short rods ranging from 0.5 to 1.0 μm. In older cultures, rods were shorter. In Fig. 2b, the PHB granules were clearly observed, and it was found that each cell contained PHB granules (about 8–10 granules) with diameters ranging from 100 to 400 nm. Butyric acid 20 g/l when used as a carbon source resulted in the production of PHB with the content of 51 wt% by *R. eutropha* stain H16 and that of 52 wt% by *R. eutropha* strain A-04. The final PHAs content in *R. eutropha* strain A-04 was different from that in *R. eutropha* strain H16 when valeric acid, γ-hydroxybutyric acid, γ-hydroxybutyric acid lactone, or 1,4-butandiol was the carbon source. However, the composition of 3HV and 4HB monomer units in P(3HB-*co*-3HV) and P(3HB-*co*-4HB) accumulated in *R. eutropha* strain A-04 was higher than that produced by *R. eutropha* strain H16. In this study, the composition of 3HB and 4HB units in the copolymer was determined by analyzing the nuclear magnetic resonance spectra. The 600 MHz ^{1}H NMR spectrum of the purified P(3HB-*co*-12%4HB) isolated from *R. eutropha* strain A-04 is shown in Fig. 3, together with the chemical shift assignment for each proton resonance. The ^{1}H NMR spectra showed that the polymers are composed of two monomer units, that is, 3HB and 4HB, and the spectrum appears at almost identical chemical shifts with those previously reported [6]. The mole fractions of 3HB and 4HB in the copolymers are calculated from the ratio of the peak areas that result from the 3HB methyl proton resonance B3 at 5.25 ppm and the 4HB methyl proton resonance A3 at 1.98 ppm. To enhance P(3HB-*co*-4HB) production, the effect of changes in the mole ratio of C/N and the ratio of mixed carbon sources on kinetic parameters for P(3HB-*co*-4HB) production were investigated in shake flask fed-batch cultivation.

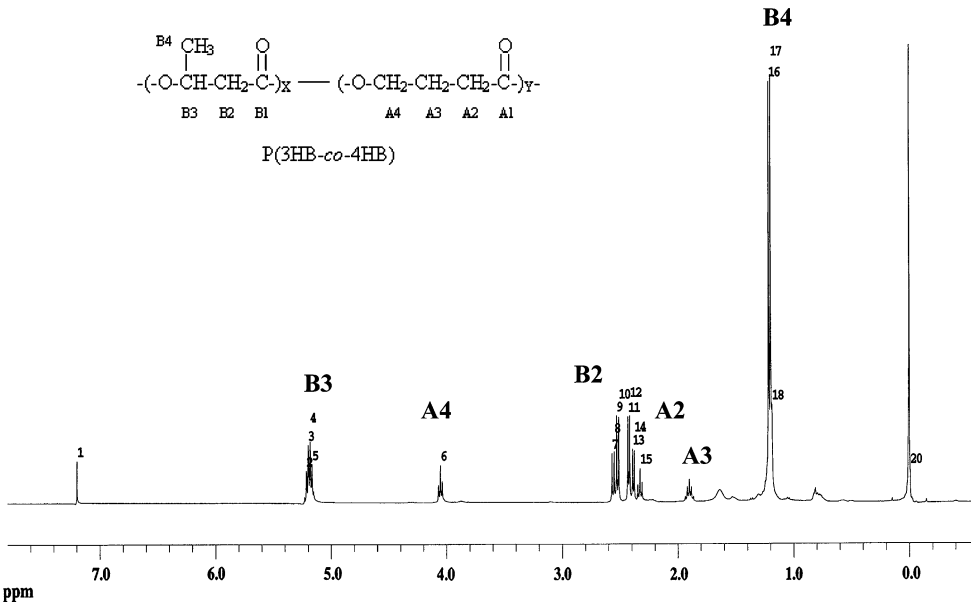

Fig. 3 600-MHz ^{1}H NMR spectra of P(3HB-*co*-12%4HB). Chemical shifts are in ppm from Me_4Si

Table 3 Production of copolyesters of P(3HB-*co*-4HB) from γ-hydroxybutyric acid and butyric acid by *R. eutropha* strain A-04 for 96 h at 30 °C

Carbon source (g/l)[a]	$(NH_4)_2SO_4$ (g/l)	No. of addition	Biomass (g/l)	PHA content (wt%)	PHA composition (mol%)	
					3HB	4HB
γ-Hydroxybutyric acid						
20 g/l	0.5	1 times	3.5	22	48	52
2 g/l	0.5	8 times	5.5	30	42	58
Butyric acid						
2 g/l	0.5	8 times	10	53	100	0
4 g/l	0.5	8 times	5	33	100	0
8 g/l	0.5	8 times	3.8	17	100	0
γ-Hydroxybutyric acid: butyric acid (50 wt%)						
3 g/l	0.5	8 times	7.4	59	86	24
6 g/l	0.5	8 times	4.3	23	56	14
9 g/l	0.5	8 times	2.1	7.6	95	5
γ-Hydroxybutyric acid: fructose (50 wt%)						
3 g/l	0.5	8 times	10.2	39	88	12
γ-Hydroxybutyric acid: butyric acid (75 wt%)						
3 g/l	0.5	8 times	6.2	48	48	42
γ-Hydroxybutyric acid: butyric acid (95 wt%)						
3 g/l	0.5	8 times	5.5	32	93	55

[a] Carbon source in production medium (100 ml, pH = 7.0)

The effect of changes in fatty acid concentrations on kinetic parameters for production of P(3HB-*co*-4HB)

In this study, γ-hydroxybutyric acid was chosen as a precursor for biosynthesis of 4HB monomer units in P(3HB-*co*-4HB) by *R. eutropha* strain A-04. The preliminary investigation was examined for promotion of P(3HB-*co*-4HB) synthesis and the results were shown in Table 3. The effect of γ-hydroxybutyric acid concentration on growth and P(3HB-*co*-4HB) production was investigated in shake flask culture either batch or fed-batch condition. Firstly, 20 g/l γ-hydroxybutyric acid and 0.5 g/l $(NH_4)_2SO_4$ were added in the production medium and it was found that biomass and P(3HB-*co*-4HB) content were

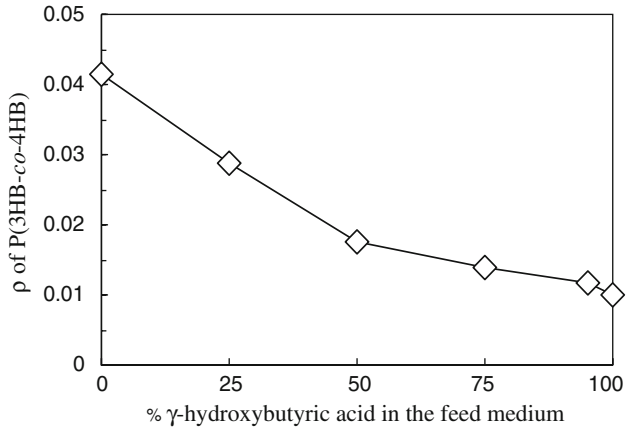

Fig. 4 The effect of the total fatty acid concentration on the specific production rate of P(3HB-*co*-4HB)

very poor. Thus, 3 g/l γ-hydroxybutyric acid and 0.5 g/l $(NH_4)_2SO_4$ was added at 12 h interval for eight times. The results showed that growth and P(3HB-*co*-4HB) production were increased. Next, a mixture of 1.5 g/l γ-hydroxybutyric acid and 1.5 g/l butyric acid and a mixture of 1.5 g/l γ-hydroxybutyric acid and 1.5 g/l fructose were compared. It was found that using a mixture of 1.5 g/l γ-hydroxybutyric acid and 1.5 g/l butyric acid gave the highest content of P(3HB-*co*-4HB). Thus, the optimum total fatty acid concentration was set at 3 g/l and the mixture of butyric acid and γ-hydroxybutyric acid was used as carbon sources in all experiments.

Next, the effect of changes in the ratio of fatty acid concentrations on the kinetic properties of P(3HB-*co*-4HB) production was investigated when the total fatty acid concentration was 3 g/l. The ratio of fatty acid was individually changed from 0, 25, 50, 75, 95, and 100% of γ-hydroxybutyric acid in the feed medium. Figure 4 shows the effect of the changes in the ratio of γ-hydroxybutyric acid in the feed medium on specific growth rate μ, and specific production rate of P(3HB-*co*-4HB) ρ, when the C/N ratio was maintained at 200. It was found that both μ and ρ decreased proportionally as the ratio of γ-hydroxybutyric acid in the feed medium increased. It was clearly observed that growth and the production of P(3HB-*co*-4HB) was inhibited by γ-hydroxybutyric acid. Thus, the effect of substrate ratio on the kinetics of P(3HB-*co*-4HB) production should be studied in details.

The effect of C/N and substrate ratio on the relationship between the specific growth rate and the specific production and the mole fraction of 4HB units in P(3HB-*co*-4HB)

Figure 5 shows the effect of change in ratios of C/N and γ-hydroxybutyric acid in the feed on the relationship between μ and ρ. In general, γ-hydroxybutyric acid is metabolized to 3HB-CoA and 4HB-CoA, which are utilized as substrates for PHA synthase in the last step of PHA synthesis [12]. By supplying γ-hydroxybutyric acid and butyric acid under various C/N ratios ranging from four to without nitrogen source, the synthesis of 4HB units was growth-associated under nitrogen-sufficient condition, and the maximum specific production rate of 4HB was obtained when C/N was set at 20. The 3HB units were highly produced under nitrogen-deficient condition. When the ratio of γ-hydroxybutyric acid in the feed was 50 wt% (Fig. 5a), the relationship had only one extreme point under nitrogen-deficient condition. In this case, the specific production rate of 4HB under nitrogen-sufficient condition was rather low. Specific production rate of 4HB increased as the concentration of γ-hydroxybutyric acid in the feed increased. It was found that when γ-hydroxybutyric acid in the feed was set at 95 wt% (Fig. 5c), ρ of the total copolymer production had locally two extreme points. One was obtained under nitrogen-deficient condition and another under nitrogen-sufficient condition. This phenomenon was explained by overlapping the relations between ρ of 3HB units versus μ and ρ of 4HB units versus μ.

The copolymers of P(3HB-*co*-4HB) exhibit wide types of polymer properties, depending on the composition of monomer units. Hence, the effect of change in C/N and substrate ratio on the mole fraction of 4HB units in the copolymer was investigated. The results are shown in Fig. 6. It should be noted that the inverse of C/N ratio was used as abscissa. The mole fraction of 4HB units increased when C/N ratio decreased. When C/N ratio decreased, which means that ammonium sulfate was oversupplied, the mole flux of acetyl-CoA reaching the TCA cycle increased and the mole fraction of 3HB units relatively decreased while the mole flux of 4HB-CoA was likely independent on the limitation of nitrogen source. It has been reported that the molar fraction of 4HB in P(3HB-*co*-4HB) biosynthesis by *R. eutropha* strain H16 was increased significantly under C/N ratio of 10 and the P(4HB) homopolymer was produced at 120 h [10]. It was suggested that in the presence of a nitrogen source, the acetyl-CoA concentration increased and the overflowing acetyl-CoA to TCA cycle seemed to cause an inhibitory effect on the ketolysis reaction catalyzing the lysis of 4-hydroxybutyratly-CoA to two molecules of acetyl-CoA, consequently, the 4HB fraction available for polymerization increased [9]. Thus, the amount of intracellular 4HB-CoA used for PHA polymerization was more than the amount of 3HB-CoA. In this study, the P(3HB-*co*-4HB) with high mole fraction of 4HB was found to be effectively produced during growth under rich nitrogen condition when the degradation of P(3HB) accumulated in the cells was occurred after 36 h. The highest mole

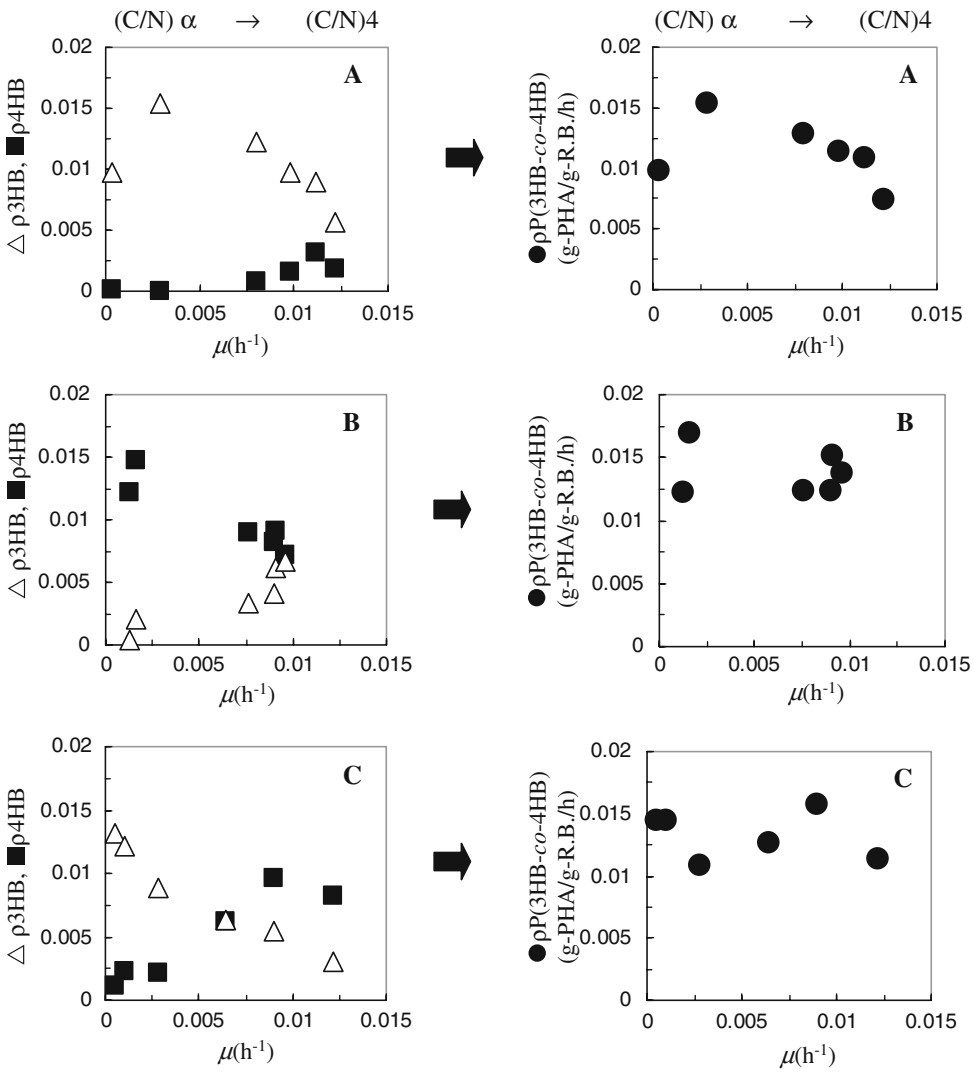

Fig. 5 The effect of C/N ratio on the relationship between μ and ρ when the ratio of γ-hydroxybutyric acid in the feed was 50 wt% (**a**), 75 wt% (**b**), and 95 wt% (**c**), respectively. *Close square* specific production rate of 4HB monomer unit, *open triangle* specific production rate of 3HB monomer unit, *close circle* specific production rate of copolymer of P(3HB-*co*-4HB)

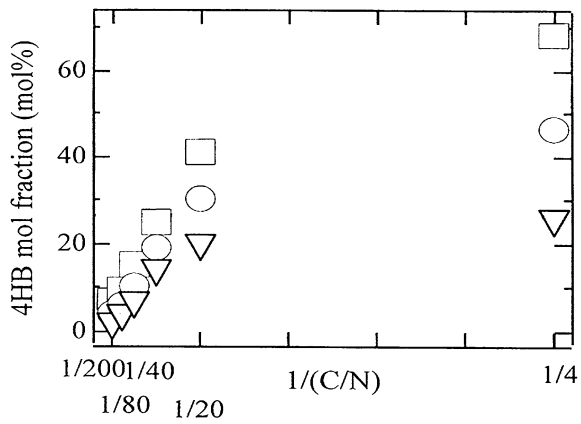

Fig. 6 The effect of C/N ratio on the mole fraction of 4HB units in P(3HB-*co*-4HB) when the ratio of γ-hydroxybutyric acid in the feed was 50 wt% (*open triangle*), 75 wt% (*open circle*), and 95 wt% (*open square*)

fraction 70 mol% could be obtained when C/N was four and the ratio of γ-hydroxybutyric acid in the feed was 95 wt%.

Development of optimal strategy for the production of P(3HB-co-4HB) based on a simple model

The optimization problem considered in this study was how to maximize the amount of P(3HB-*co*-4HB) produced within a final cultivation time. The dynamics of the residual biomass concentration, X_r, specific growth rate μ, P(3HB-*co*-4HB) concentration, P, and specific production rate ρ, in batch or fed-batch cultivation can be represented as [17]:

$$\frac{dVX_r}{dt} = \mu VX_r \quad (1)$$

$$\frac{dVP}{dt} = \rho VX_r \quad (2)$$

where V, X_r, and P are volume of the medium, residual biomass concentration and P(3HB-*co*-4HB) concentration, respectively. At the final cultivation time, t_f the performance index, J was given as the total production amount of $VP(t_f)$ for maximum production of P(3HB-*co*-4HB) and can be represented as [19]:

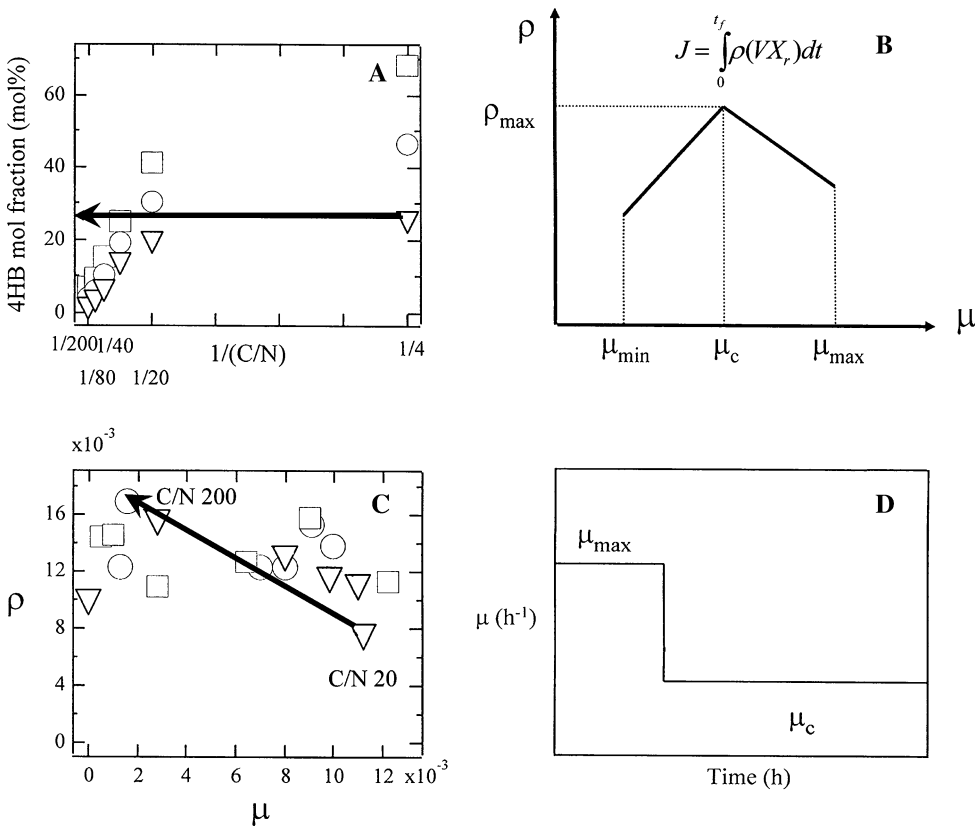

Fig. 7 The strategy for maximum of P(3HB-*co*-4HB) production. **a** The target mole fraction of 4HB unit was set at 28 mol% as indicated by *solid arrow*. **b** The optimal solution solved by Pontryagin's maximum principle based on the relationship between μ and ρ. **c** The summary of experimental results under various C/N when γ-hydroxybutyric acid in the feed was 50 wt% (*open triangle*), 75 wt% (*open circle*), and 95 wt% (*open square*) and the *solid arrow* indicates movement of the switching of C/N and substrate ratio. **d** Optimal profile of μ employed in PHA production

$$J = \int_0^{t_f} \rho(VX_r)\mathrm{d}t \tag{3}$$

The optimal profile of μ employed in PHA production was exactly solved as described previously [3]. The optimal profile of μ in PHA production was demonstrated in Fig. 7. However, in this study, when a nitrogen source was added to the production medium under nitrogen sufficient condition after adequate P(3HB-*co*-4HB) accumulation in the cells, not only P(3HB-*co*-4HB) production but also 3HB degradation occurred simultaneously. There have been reported that re-utilization of 3HB was naturally occurred when the substrate was exhausted in the presence of nitrogen source [18]. Thus, the prevention of 3HB degradation needs to be included in the optimization problem. In this method, the relationship between μ and ρ under various C/N and substrate feeding ratios was used, as summarized and shown in Fig 7c. It was found that μ and ρ could be changed by manipulating C/N and substrate feeding ratios. As shown in Fig. 7b, not only the ratio of γ-hydroxybutyric acid in the feed but also the mole ratio of C/N in the medium very much affected the relationship between μ and ρ. Among these conditions, μ was maximum when γ-hydroxybutyric acid in the feed was set at 50 wt%. Therefore, to implement the optimal strategy easily in the cultivation, the boundary control of μ and the switching of both the ratios of substrate and C/N were done as follows: in the growth stage, μ was kept at a maximum value, $\mu_{\max}$, and in the production stage, ρ was kept at a maximum value. In this study, the final cultivation time, t_f was set at 96 h. The optimal switching time of μ, t_c, was determined by

$$t_c = t_f - \frac{\ln\left(\frac{b}{\rho_{\max}}\right)}{\mu_c} \tag{4}$$

where b corresponds to the intercept of linear line plotted in Fig 7c, respectively. The summary of this strategy is shown in Table 4. The study of optimal production was successfully realized by the experiments, as shown in Fig. 8. In Fig 8a, the open circle represented time courses of P(3HB-*co*-4HB) production under control cultivation when the mole ratio of C/N was kept constant at 20 and ratio of γ-hydroxybutyric acid was set at 50 wt%. The content of PHA reached a saturation value of 55 wt% after 36 h with 4HB mole fraction unit 33 mol%. The degradation of 3HB was occurred after 36 h whereas 4HB mole fraction unit was produced simultaneously. In Fig 8b, the open triangle is a result when the mole ratio of C/N was kept constant at 20, but the ratio of γ-hydroxybutyric acid was switched from 50 to 25 wt% to supply more butyric acid for promotion of 3HB synthesis. The total PHA content was 60 wt% with 4HB mole fraction unit 25 mol%. In Fig 8c, the closed triangle represents a result when the ratio of

Table 4 Summary of the strategy for maximum of P(3HB-*co*-4HB) production at the given final cultivation time

	Growth phase (0–36 h)	Production phase (36–96 h)
Case 1		
Control experiment		
μ	μ_{max}	μ_{max}
Ratio of C/N	20	20
Ratio of γ-hydroxybutyric acid (wt%)	50	50
Case 2		
Switching ratio of γ-hydroxybutyric acid		
μ	μ_{max}	μ_{max}
Ratio of C/N	20	20
Ratio of γ-hydroxybutyric acid (wt%)	50	25
Case 3		
Switching ratio of (C/N)		
μ	μ_{max}	μ_c
Ratio of C/N	20	200
Ratio of γ-hydroxybutyric acid (wt%)	50	50
Case 4		
Optimal condition		
μ	μ_{max}	μ_c
Ratio of C/N	20	200
Ratio of γ-hydroxybutyric acid	50	25

γ-hydroxybutyric acid was set at 50 wt%, but the ratio of (C/N) was switched from 20 to 200 to cut off the nitrogen source after switching time. The content of P(3HB-*co*-4HB) was 65 wt% with 4HB mole fraction unit 29 wt%. In Fig 8d, the closed circle shows the production when the optimal strategy was employed. Finally, the productivity could be enhanced until the final cultivation time. The total PHA content was 71 wt% with mole fraction of 4HB unit 30 mol% in the copolymer.

Conclusions

The newly isolated bacterium strain A04 was identified as *R. eutropha* and was named *R. eutropha* strain A-04. The experiments performed in this study suggest that *R. eutropha* strain A-04 could utilize various carbon sources for growth and production of P(3HB-*co*-4HB). It was found that the incorporation of 4HB is dependent on the use of precursors such as γ-hydroxybutyric acid and 1,4-butanediol [12]. The synthesis of 4HB units was growth-associated under nitrogen-sufficient condition. The synthesis of 3HB units was enhanced under nitrogen-deficient condition. The overlapping of these relations between the specific production rate of 3HB versus μ and those of 4HB units versus μ gave the specific production rate of P(3HB-*co*-4HB) versus μ, and it had two locally extreme points with different mole fractions of 4HB units. The mole fraction of 4HB units of P(3HB-*co*-4HB) increased as C/N decreased. The highest mole fraction of 4HB units was obtained when C/N was 4. The mole fraction of 4HB units increased proportionately as the ratio of γ-hydroxybutyric acid in the feed increased at any value of C/N. Thus, the mole fraction of 4HB units can be changed from 0 to 70 mol% by adjusting the ratio of substrate and the mole ratio of C/N. Finally, the optimal strategy for maximum

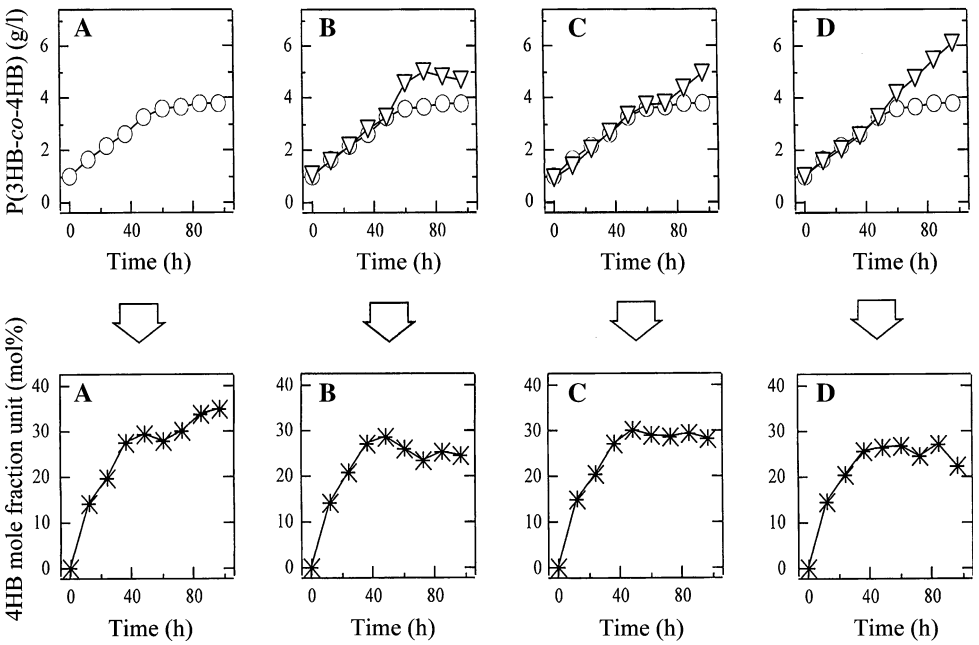

Fig. 8 Experimental comparison of P(3HB-*co*-4HB) production by the optimal strategy with that by the constant control strategy. **a** *Open circle* constant control strategy. **b** *Open triangle* optimal strategy when the C/N ratio was kept at 20 and the ratio of γ-hydroxybutyric acid switched from 50 to 25 wt%. **c** *Open triangle* optimal strategy when the ratio of γ-hydroxybutyric acid was kept constant at 50 wt% and the C/N ratio was switched from C/N 20 to C/N 200. **d** *Open triangle* optimal strategy when the C/N ratio was switched from C/N 20 to C/N 200 and the ratio of γ-hydroxybutyric acid switched from 50 to 25 wt%. *Asterisk* the mole fraction of 4HB unit

production of P(3HB-*co*-4HB) was obtained, and was successfully realized by experiments. Therefore, *R. eutropha* strain A-04 possesses a high potential for the production of P(3HB-*co*-4HB).

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