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Effect of C:N Molar Ratio on Monomer Composition of Polyhydroxyalkanoates Produced by *Pseudomonas mendocina* 0806 and *Pseudomonas pseudoalkaligenus* YS1

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Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by bacteria. In this study, the effect of C:N molar ratio on the monomer composition of PHAs was investigated, including medium chain length PHA produced by *Pseudomonas mendocina* 0806 and PHA blends consisting of monomers of 3-hydroxybutyrate and medium chain length hydroxyalkanoate produced by *Pseudomonas pseudoalkaligenus* YS1. It was observed that there were some fixed ranges of C:N molar ratio that affect the monomer composition of PHA independently of the substrate. For strain 0806, the ranges were C:N < 20, 20 < C:N < 200, and C:N > 200. The monomer composition was constant among these ranges when using glucose and octanoate as the sole substrate. For strain YS1, the ranges were C:N < 20, 20 < C:N < 45, and C:N > 45. These results are useful for controlling monomer composition in PHA production.

Index Entries: Polyhydroxyalkanoates; polyhydroxybutyrate; *Pseudomonas mendocina*; *Pseudomonas pseudoalkaligenus*.

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Introduction

Polyhydroxyalkanoates (PHAs) are intracellular polymers stored by many bacteria *(1)*. They have attracted increasing attention in the scientific community, because they are polymers with characteristics of biodegradability, biocompatibility, and optical activity *(1,2)*.

Polyhydroxybutyrate is a homopolymer of 3-hydroxybutyrate (HB) and is the most widespread and best-characterized member of the PHAs. Other PHAs are heteropolymers consisting of various monomer units, including copolymers consisting of short chain length monomers (3–5 carbon atoms) and medium chain length monomers (6–16 carbon atoms) *(1)*. They have properties similar to thermoplastics and elastomers, depending on the monomer structure *(3)*. Apart from the PHAs just mentioned, the third type of PHAs is blends of medium chain length hydroxyalkanoate (HA) and short chain length HB that have been found to be accumulated in *Pseudomonas* sp. 61-3 *(3,4)*, *Pseudomonas* sp. A33 *(5)*, and recombinant *Pseudomonas (6)*.

Because the properties of polymers are greatly dependent on monomer composition, it is important to choose monomer compositions for various PHA applications. Some factors are known to affect the monomer composition of PHAs. First, the composition depends on the PHA synthase. The most studied PHA synthase, found in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), has been shown to produce polymers containing monomer units limited to C3 to C6 in *R. eutropha* and when cloned to other bacterial strains *(7)*, whereas, the fluorescent Pseudomonads belonging to the rRNA homology group I accumulate PHAs consisting mostly of medium chain length hydroxy-fatty acids *(8)*. Second, the composition depends on the carbon source. For example, some bacteria can incorporate 3-hydroxypropionic acid, 4-hydroxybutyric acid, or 5-hydroxyvaleric acid into the polyester when suitable precursors are provided *(2,8)*. Saturated and unsaturated aliphatic molecules consisting of up to 10 carbon atoms, branched chain aliphatic molecules, cyclic aliphatic or aromatic side chains, and aliphatic molecules with functional groups can serve as building blocks for PHA polymers *(2)*.

Most bacteria accumulate PHAs when their growth is limited by an essential nutrient, such as N, P, Mg, K, O, or S in the presence of an excessive carbon supply *(8)*. Nitrogen limitation is considered an important factor that affects PHA accumulation, cell dry weight, and polymer content during PHA production *(4,9)*.

In one of our previous studies, *Pseudomonas mendocina* 0806 was found to produce medium chain length PHA. The structure and the properties of the polymer have been investigated *(10)*, and the effects of carbon and nitrogen sources on PHA production have also been studied *(11)*. *Pseudomonas pseudoalkaligenous* YS1 was isolated and found to accumulate short and medium chain length PHA blends. We observed that the monomer composition of these two types of PHA produced by the two strains was affected

by C:N molar ratio in the media. We report herein that C:N molar ratio can have a significant effect on the composition of PHA heteropolymer and will be useful for regulation of monomer composition during PHA production.

Materials and Methods

Microorganisms and Cultivation Conditions

Strain 0806 was isolated from an oil-contaminated river near an operating oil field in Tianjin City, northern China, and was identified as a species of *P. mendocina* by the Institute of Microbiology, Academia Sinica, China. Strain YS1 was isolated from a wastewater treatment plant, Yanshan Petroleum, Beijing, and was identified as a species of *P. pseudoalkaligenus* by the Institute of Microbiology.

Media containing the following minerals were used: 3.8 g/L of Na_2HPO_4, 2.65 g/L of KH_2PO_4, and 0.2 g/L of $MgSO_4$, supplemented with a 0.1% microelement solution containing per liter of 0.1 *N* HCl 0.218 g of $CoCl_2$, 9.7 g of $FeCl_3$, 7.8 g of $CaCl_2$, 0.118 g of $NiCl_3$, 0.105 g of $CrCl_3 \cdot 6H_2O$, and 0.156 g of $CuSO_4 \cdot 5H_2O$. To study the effect of C:N ratio on the monomer composition of PHA of *P. mendocina* 0806, 20 g/L of glucose and 0.1–8.1 g/L of $(NH_4)_2SO_4$, and 4 g/L of octanoate and 0.0065–0.52 g/L of $(NH_4)_2SO_4$ were used to obtain C:N ratios from 5 to 400. To study the effect of C:N ratio on monomer composition of PHA produced by strain YS1, 8 g/L of octanoate and 0.035–2.08 g/L of $(NH_4)_2SO_4$ were used to obtain C:N ratios from 10 to 150, and 1–9 g/L of octanoate and 0.5 g/L of $(NH_4)_2SO_4$ were used to study the effect of octanoate concentration on PHA monomer composition. Five milliliters of culture grown overnight in nutrient broth for strain 0806 and 5 mL of culture grown overnight in 8 g/L of octanoate mineral medium plus 1 g/L of peptone and 0.5 g/L of beef extraction for strain YS1 were inoculated in 500-mL shake flasks containing 100 mL of the previously given media and were incubated on a rotary shaker (NBS, Series 25D; New Brunswick), at 200 rpm and 30°C for 48 h.

The cells were harvested by centrifugation at 5000*g* for 10 min, and the PHAs were extracted using a combination of sodium hypochlorite and chloroform as described by Hahn et al. *(12)*. The cell dry weight and PHA dry weights were determined as described by Page *(13)*.

PHA Analysis

Polyesters in dried cells or in purified forms were methyl esterified in a mixture of chloroform and methanol-sulfuric acid as described by Braunegg et al. *(14)*. The lower chloroform solution was collected for analysis, using a gas chromatogram (Beifen, SQ-204; Beijing, China) equipped with a column of Chromosorb 101 treated with polyethylene glycol 20000 (packed by the Institute of Microbiology). Various 3-HA methyl esters, including HB, 3-hydroxyhexanoate (HHx), 3-hydroxyoctanoate (HO), and 3-hydroxydecanoate (HD), were purchased from Sigma (St. Louis, MO),

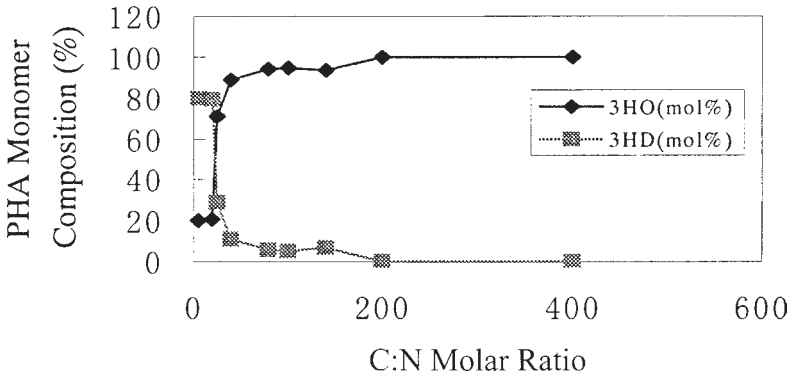

Fig. 1. Effect of C:N molar ratio on monomer compositions of medium chain length PHA produced by strain *P. mendocina* 0806 using octanoate as a sole carbon source.

and a PHA standard sample with known monomer contents of HHx, 3-hydroxyheptanoate or C_7, HO, 3-hydroxynonanoate or C_9, HD 3-hydroxy-undecanate or C_{11}, and 3-hydroxy-5-dodecanoate or $C_{12}\Delta_5$ (HDD) were used as standards to determine the polyester compositions both quantitatively and qualitatively, based on the areas and retention times of individual HA monomers on the chromatogram.

Results and Discussion

Effect of C:N Molar Ratio on Monomer Composition of PHA Produced by P. mendocina *0806 Using Octanoate and Glucose as Sole Carbon Source*

P. mendocina 0806 was found to accumulate medium chain length PHA from a related carbon source, namely octanoate, and from an unrelated carbon source such as glucose or citric acid *(11)*.

When using 4 g/L of octanoate as carbon source and changing the concentration of $(NH_4)_2SO_4$ to get a C:N molar ratio of 5–400, it was observed that there was only 20% HO monomer in PHA when the C:N molar ratio was below 20, whereas it increased to nearly 100% as C:N molar ratio reached above 200. On the other hand, the percentage of HD monomer decreased from 80% to 0 as C:N ratio increased from 5 to 200. The percentage of HO and HD monomers remained almost constant at 71–95 and 29–5%, respectively, at a C:N ratio of 20–200 (Fig. 1). As the C:N ratio increased, cell dry weight decreased. The highest PHA yield was at a C:N ratio of 80 and the highest PHA content was at a C:N ratio of 140 (Fig. 2).

When glucose served as the substrate, HD was the major monomer in PHA reword this HO was the major monomer when octanoate served as the substrate. There was nearly 100% of HD monomer with 0.4% of HB monomers, and no HO or HDD monomer when the C:N ratio was below 20. HO and HD monomer appeared when the C:N ratio was above 20, but no

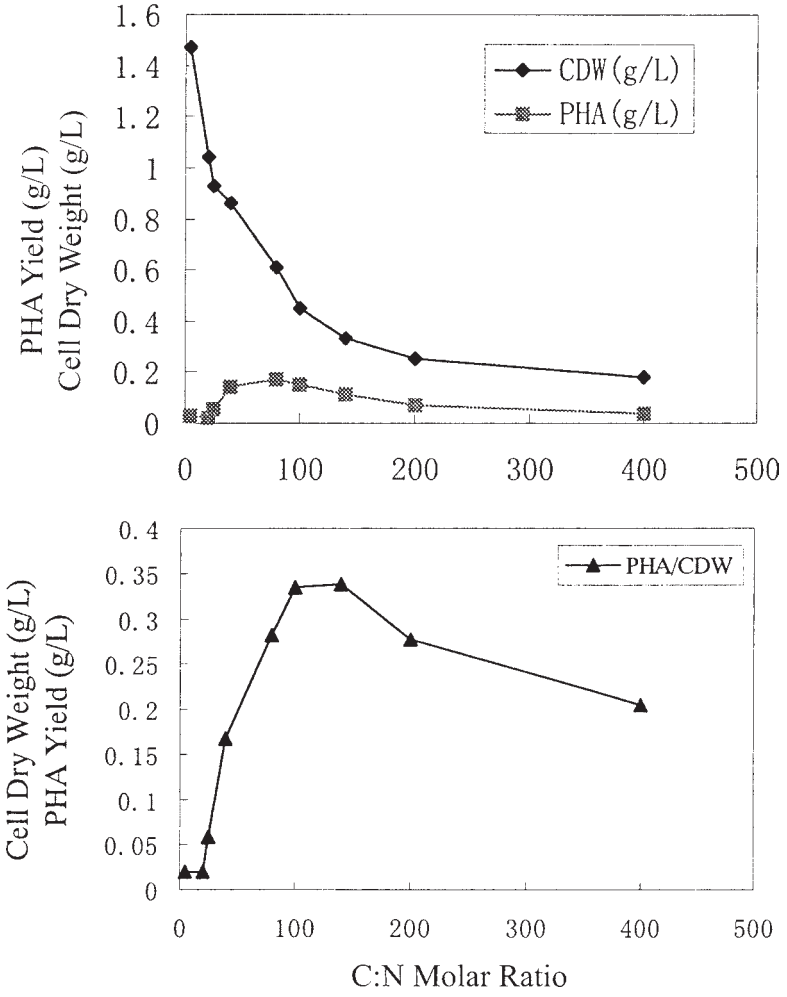

Fig. 2. Effect of C:N molar ratio on PHA production by strain *P. mendocina* 0806 using octanoate as carbon source, showing the changes of cell dry weight, PHA yield, and PHA content (PHA/CDW). CDW, cell dry weight.

HB monomer was observed. The percentage of HO monomer remained constant at 12–11 at a C:N ratio of 20–200. HD monomer decreased and HDD monomer increased when the C:N ratio was above 200 (Fig. 3). Also, similar to using octanoate as substrate, the cell dry weight decreased when the C:N ratio was increased, and the maximum PHA yields and PHA content was observed at a C:N ratio of 40 and 200, respectively (Fig. 4).

Comparing the effect of C:N ratio on monomer composition of PHA synthesized from octanoate and glucose, we found that there was a fixed range for various monomer compositions, which seemed independent of the substrate. The ranges could be divided into C:N ratio < 20, 20 < C:N ratio < 200, and C:N ratio > 200. Within these ranges, the monomer compositions were maintained at a constant level, as shown in Table 1.

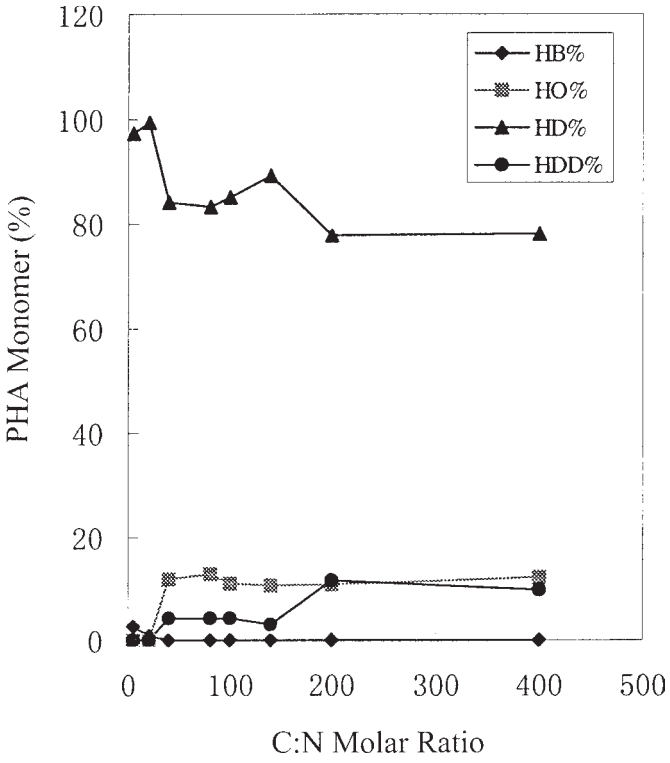

Fig. 3. Effect of C:N molar ratio on monomer composition of medium chain length PHA produced by strain *P. mendocina* 0806 using glucose as the sole carbon source.

Effect of C:N Molar Ratio on Monomer Composition of PHA Produced by P. pseudoalkaligenus *YS1*

P. pseudoalkaligenus YS1 was found to produce blends of medium chain length HA and short chain length HB from related carbon sources such as octanoate and myristic acid. It could not accumulate PHA from unrelated substrate such as glucose (unpublished data). To investigate the influence of C:N ratio on the PHA blends produced by the strain *P. pseudoalkaligenus* YS1, the $(NH_4)_2SO_4$ concentration was changed in the presence of 8 g/L of sodium octanoate. The results showed that the C:N ratio affected the PHA composition dramatically (Table 2). First, it influenced the monomer type: when C:N was 10, only HB monomer was synthesized; medium chain length monomer was synthesized at a C:N ratio of 25. Second, it influenced the monomer molar ratio: the proportion of HB to HO was >1 when C:N < 50, and <1 at C:N > 50; the proportion of HB, HO, and HD was constant when the C:N ratio was >50. As the C:N ratio increased from 25 to 55, the HB monomer increased and medium chain length monomer decreased. PHA containing 82–95% HB monomer was obtained when the C:N ratio was at 25–35. It has been reported that PHA containing 85–95% HB and some medium chain length HA monomer showed better processing property compared with that of other PHAs *(8)*.

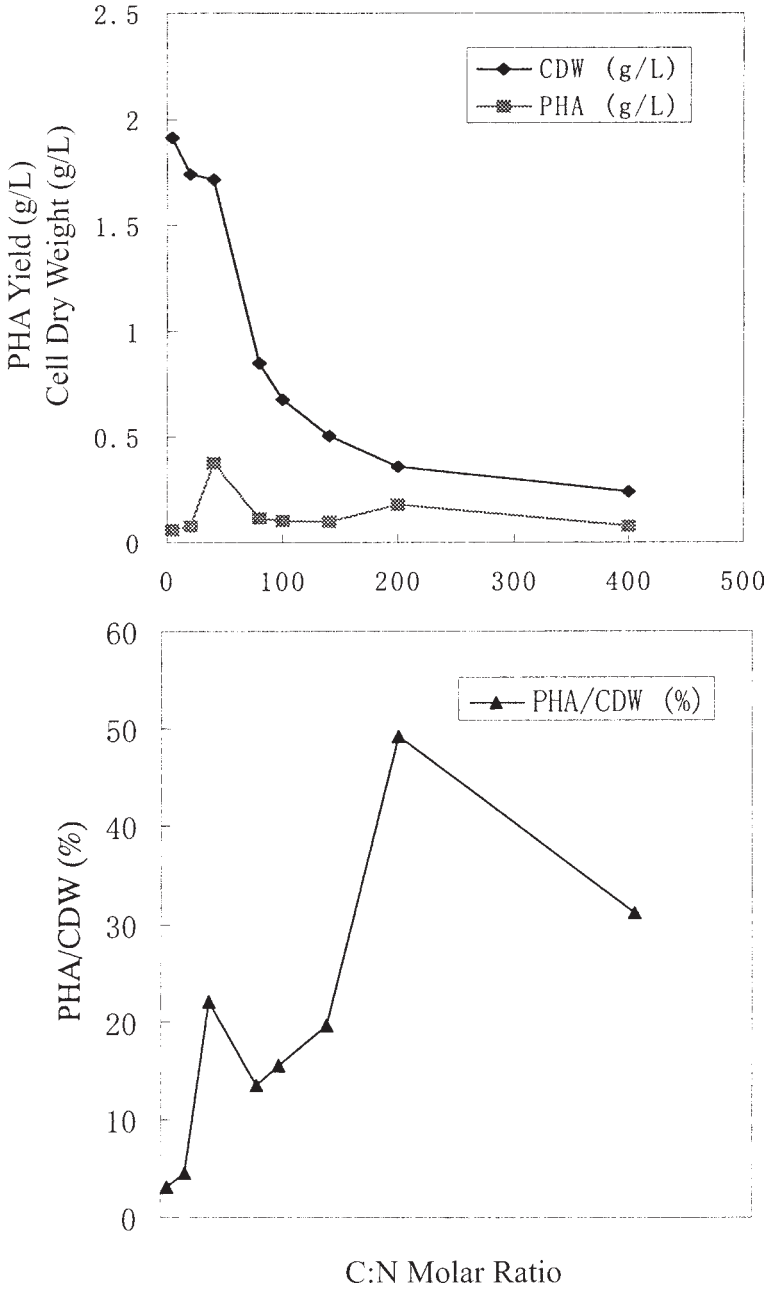

Fig. 4. Effects of C:N molar ratio on PHA production of strain *P. mendocina* 0806, showing the changes of cell dry weight, PHA yield, and PHA content (PHA/CDW).

To investigate the influence of octanoate concentration on the PHA monomer composition produced by strain YS1, the concentration of $(NH_4)_2SO_4$ was fixed at 0.5 g/L, and concentrations of octanoate were changed. The results reflected the effect of C:N ratio on PHA composition.

Table 1
Effect of C:N Molar Ratio on Monomer Composition of Medium Chain Length PHA Produced by Strain 0806 Using Glucose and Octanoate as Sole Carbon Sources

		PHA Monomer composition (%)			
Carbon source	C:N molar ratio	HB	HO	HD	HDD
Octanoate	<20	—	20–21	80–79	—
	20–200	—	71–95	29–5	—
	>200	—	100	—	—
	<20	3–1	—	97–99	—
Glucose	20–200	—	12–11	84–89	4–0
	>200	—	11–12	78	12–10

Table 2
Effect of C:N Molar Ratio on PHA Monomer Composition Produced by *P. pseudoalkaligenus* YS1 Grown in 8 g/ L of Octanoate

		PHA composition (%)		
C:N molar ratio	$(NH_4)SO_4$ (g/L)	HB	HO	HD
10	2.5	100	—	—
25	1.0	95	4.7	0.3
35	0.71	82	16.7	1.3
45	0.55	52	46	2
50	0.5	35	61	4
55	0.45	29	68	3
65	0.38	30	67	3
75	0.34	32	65	3
100	0.25	36	60	4
125	0.2	31	65	4
150	0.7	31	66	3

When octanoate was <3 g/L, the C:N ratio was <20; only HB monomer was synthesized. When octanoate was >3 g/L, and the C:N ratio was >20, medium chain length monomer was synthesized. When C:N > 45, HB and HO monomer maintained constant at 77–72% and 23–28%, respectively. These results agreed with those obtained when the octanoate concentration was fixed and $(NH_4)_2SO_4$ concentration was changed (Table 3).

The effect of C:N molar ratio on monomer composition of PHA produced by strain YS1 might be in the following three ranges: C:N < 20, 20 < C:N < 45, and C:N > 45. Table 4 gives the monomer composition maintained at a constant level among these ranges.

The results obtained showed that C:N molar ratio affected monomer compositions of PHAs. It was independent of the type and concentration of the substrates. There were three ranges of C:N molar ratio for the accumulation of PHAs of the two investigated strains. For *P. mendocina* 0806, which

Table 3
Effect of Octanoate Concentration on Monomer Composition of PHA Produced by *P. pseudoalkaligenus* YS1

Sodium octanoate (g/L)	C:N molar ratio	PHA composition (%) HB	HO
1	6.25	100	—
2	12.5	100	—
3	18.75	98	2
4	25	96	4
5	31.25	92	8
6	37.5	89	11
7	43.75	77	23
8	50	77	23
9	56.25	72	28

Table 4
Effect of C:N Molar Ratio on Monomer Composition of PHA Produced by Strain YS1 When Changing Concentration of $(NH_4)_2SO_4$ or Octanoate

	C:N molar ratio	PHA composition (%) HB	HO	HD
Concentration of $(NH_4)_2SO_4$ changed while octanoate fixed	C:N < 20	100	—	—
	20 < C:N < 45	95–82	4.7–16.7	0.3–1.3
	C:N > 45	35–31	61–66	4–3
Concentration of $(NH_4)_2SO_4$ fixed while octanoate changed	C:N < 20	100	—	—
	20 < C:N < 45	98–89	2–11	—
	C:N > 45	77–72	23–28	—

produces medium chain length PHA, the ranges were C:N > 20, 20 < C:N < 200, and C:N > 200, whenever using either octanoate or glucose as the carbon source. For strain *P. pseudoalkaligenus* YS1, which produces short and medium chain length PHA blends, the ranges were C:N < 20, 20 < C:N < 45, and C:N > 45, whenever changing either carbon or nitrogen concentrations.

It was also observed that C:N molar ratio influenced on the PHA yield and cell dry weight. This finding agrees with the results of Kato et al. *(4)* on PHA blends produced by *Pseudomonas* sp. 61-3 *(4)* and the PHA accumulation in activated sludge biomass reported by Chua et al. *(9)*.

It has previously been reported that substrate or precursor influenced monomer composition of PHAs *(2,15)*, and that C:N ratio affected cell dry weight. Our results showed that the C:N ratio can also affect the monomer composition of PHAs, especially PHA heteropolymers. It is useful for controlling constant C:N ratio when a certain percentage of monomers is desired to obtain a proper melt temperature, crystallinity, and stiffness of the polymer *(16)*.

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