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Cometabolic Production of Poly(3-Hydroxyalkanoates) Containing Carbon-Carbon Double and Triple Bonds by *Pseudomonas oleovorans*

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Abstract Poly(3-hydroxyalkanoate) copolyesters containing both carbon-carbon double and carbon-carbon triple bonds were produced by *Pseudomonas oleovorans* grown in mixtures of 10-undecynoic acid (10-UND(≡)) and 10-undecenoic acid (10-UND(=)). The PHA content in the dry cells was usually 40 wt%. The bioconversion yield of 10-UND(≡) to PHA by *P. oleovorans* was remarkably enhanced from 1% to over 24% as the fraction of 10-UND(=) in the carbon substrate mixtures increased from 0 to 50%. These values were higher than those obtained when *P. oleovorans* was grown in the same molar mixtures of 10-UND(≡) and nonanoic acid (NA), indicating that 10-UND(=) was more efficient than NA as a cosubstrate in inducing cometabolic PHA production.

Key words: Cometabolism, poly(3-hydroxyalkanoate), *Pseudomonas oleovorans*, unsaturated repeating units

Of the microorganisms capable of producing medium-chain-length poly(3-hydroxyalkanoates) (mcl-PHAs), *Pseudomonas oleovorans* and *P. putida* have been most intensively investigated. These microorganisms can produce mcl-PHAs containing various functional groups, such as halogens [2, 10] cyclohexyl [8], aromatic [5, 12, 17, 18], and unsaturated [3, 6, 11] groups in the side chains. PHAs containing such unusual functional groups have been produced through cometabolism, that is, these PHAs have only been produced when the microorganisms are grown in mixtures including a carbon substrate bearing the specific functional group and good PHA-producing carbon substrate, such as octanoic acid (OA) and nonanoic acid (NA) [7, 10, 15].

PHAs containing unsaturated repeating units are of great interest, because polymers with different properties and functional groups can be prepared through chemical modifications of these groups. Unsaturated repeating units are also useful as crosslinking centers with photochemical and thermal radical reactions [9]. Some chiral (*R*)-3-hydroxycarboxylic acids obtained from unsaturated PHAs and modified PHAs are also expected to be useful as starting materials for new biomaterials [13].

PHAs containing either carbon-carbon double (C=C) bonds or carbon-carbon triple (C≡C) bonds have already been prepared using *P. oleovorans* [6, 11] and *P. putida* [6,7] grown with carbon substrates containing appropriate unsaturated groups. In most previous studies, 10-undecynoic acid (10-UND(≡)) and 10-undecenoic acid (10-UND(=)) have been used as the carbon source that provides C≡C bonds and C=C groups, respectively. Recent results concerning PHA production from 10-UND(≡) showed that the PHA contents in *P. putida* grown with 10-UND(≡) were lower than 4% of the dry cell weight [6]. The current authors are interested in increasing the PHA yield from 10-UND(≡), considering the potential application of PHAs containing C≡C bonds and chiral (*R*)-3-hydroxyalkynoates that can be obtained from these polyesters. Accordingly, the present study examined the production of PHAs by *P. oleovorans* grown with mixtures of 10-UND(=) and 10-UND(≡) in order to prepare PHAs containing both C=C bonds and C≡C bonds, and to elucidate whether 10-UND(=) induces the conversion of 10-UND(≡) to PHA with a higher yield.

P. oleovorans ATCC 29347 was grown on a basal medium [14], which contained NA, 10-UND(=), and 10-UND(≡) as the carbon substrates for growth and PHA production. The batch fermentation experiments were carried out using a 5-l jar fermentor with a working volume of 3 l. The medium was inoculated with 3% (v/v) inoculum in an overnight culture in the basal medium, which contained 10 mM glucose as the sole carbon source. The temperature and pH were automatically controlled at optimal values of 30°C and 7.0, respectively. The airflow rate and stirring speed was 0.25 vvm and 250 rpm, respectively. The cell growth was monitored spectrophotometrically at 660 nm. The fermentation was stopped approximately 2 h after the

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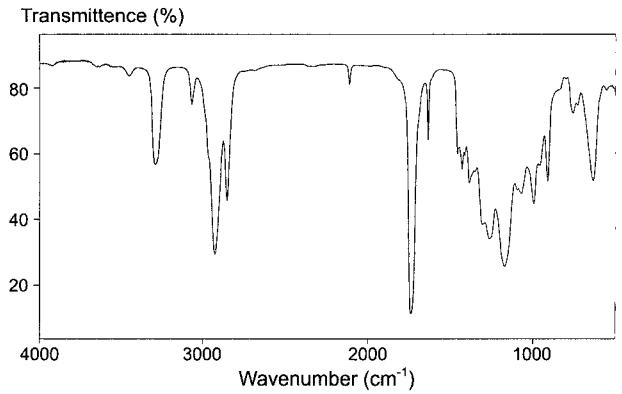

Fig. 1. IR spectrum of the PHA produced by *P. oleovorans* from an equimolar mixture of 10-UND(=) and 10-UND(≡): =C-H stretch (3,072 cm^{-1}), C=C stretch (1,638 cm^{-1}), ≡C-H stretch (3,291 cm^{-1}), and C≡C stretch (2,116 cm^{-1}).

growth reached the stationary phase, then the cells were harvested by centrifugation followed by lyophilization. The PHAs were routinely isolated and purified from the lyophilized cells with hot chloroform using a Soxhlet apparatus [1]. The relative concentration of PHA monomeric units synthesized was calculated by integrating the methyl-ester peaks from the methanolyzed samples using gas chromatography [7]. Identification of the PHA monomeric units was determined by either gas chromatography/mass spectrometry (GC/MS) or ^1H nuclear magnetic resonance spectroscopy. Differential scanning calorimetry (DSC), infrared (IR) spectrometry, and gel permeation chromatography (GPC) analyses were performed, as previously described [12].

Figure 1 shows the IR spectrum of the PHA synthesized by *P. oleovorans* grown in an equimolar mixture of 10-UND(=) and 10-UND(≡), showing characteristic absorption peaks of both a C=C bond and a C≡C bond at 3,072 and 1,638 cm^{-1}, and 3,291 and 2,116 cm^{-1}, respectively.

The composition of the PHAs obtained from *P. oleovorans* grown in various carbon substrate mixtures is shown in Table 1. The PHAs produced from mixtures of 10-UND(=) and 10-UND(≡) contained 3-hydroxy-8-nonenoate, 3-hydroxy-8-nonynoate, 3-hydroxy-6-heptenoate, 3-hydroxy-10-undecenoate, and 3-hydroxy-10-undecynoate units. It was remarkable that no 3-hydroxy-6-heptynoate unit was detected in any PHA, even though this unit could have been produced by the deacetylation of 3-hydroxy-8-nonynoate. Considering that both 3-hydroxyheptanoate and 3-hydroxy-6-heptenoate have been previously incorporated into PHAs by *P. oleovorans*, the absence of 3-hydroxy-6-heptynoate in the current PHAs suggested that a straight ethynylmethyl group in 3-hydroxy-6-heptynoate prevented this molecule from being polymerized. It has also been reported that repeating units containing alkyl substituents at carbon 6 were not found in PHAs [4]. However, according to recent reports [5, 12, 18], even shorter monomers, such as 3-hydroxy-5-phenylvalerate and 3-hydroxy-4-phenylbutyrate as well as monomer units with a more bulky group, such as phenyl and phenoxy at carbon 6, can be incorporated into the polymer chain in *Pseudomonas* spp. Therefore, it is unclear why repeating units containing carbon-carbon triple bonds or alkyl substituents at carbon 6 were not incorporated into the growing polymer chain.

Table 1 also shows that the glass transition of the PHAs increased as the amount of C≡C bonds increased and decreased as the amount of C=C bonds increased. These results can be explained by the higher and lower symmetry of the repeating units caused by the C≡C and C=C bonds, respectively. These results are in good agreement with previous results obtained by the current authors for PHAs containing 3-hydroxyalkanoate and either 3-hydroxyalkenoate or 3-hydroxyalkynoate units [6, 11].

No melting endotherm was observed in the DSC thermograms of the PHAs obtained in this study. The smooth change in the glass transition temperatures of the polymers indicated that these PHAs were random copolyesters. Different fractions obtained from repeated fractionation of the PHA from an equimolar mixture of 10-

Table 1. Relative concentration of monomer units in PHAs produced by *P. oleovorans* grown with various mixtures of NA, 10-UND(=), and 10-UND(≡).

Conc. of carbon substrate (mM)			Relative concentration of monomer units in PHAs (%)[a]							T_g (°C)
NA	10-UND(=)	10-UND(≡)	3HHp(=)	3HHp	3HN(=)	3HN	3HN(≡)	3HUD(=)	3HUD(≡)	
	2.5	7.5	6.0		16.2		55.1	5.0	17.7	-34.2
	5	5	8.6		29.6		39.8	8.8	13.2	-39.1
	7.5	2.5	13.6		46.7		16.4	16.2	7.1	-44.0
5	5	5	8.2	10.9	21.0	17.3	23.5	9.3	9.8	-37.5
10	5	5	5.1	16.2	16.4	28.9	19.2	6.3	7.9	-37.6
5	10	5	6.4	6.5	34.0	15.7	16.6	13.2	7.6	-43.5
5	5	10	5.1	9.2	16.3	13.9	35.8	6.1	13.6	-39.3

[a]GC area %.

3HHp(=), 3-hydroxy-6-heptenoate; 3HHp, 3-hydroxyheptanoate; 3HN(=), 3-hydroxy-8-nonenoate; 3HN, 3-hydroxynonanoate; 3HN(≡), 3-hydroxy-8-nonynoate; 3HUD(=), 3-hydroxy-10-undecenoate; 3HUD(≡), 3-hydroxy-10-undecynoate.

UND(≡) and 10-UND(=) using different amounts of a good solvent, chloroform, and poor solvent, methanol, had similar compositions, also indicating that these PHAs were random copolyesters.

In the present work, it was found that the bioconversion yield of 10-UND(≡) to PHA by *P. oleovorans* and the PHA content in the cells were 1% and 3 wt%, respectively, when *P. oleovorans* was grown solely with 10-UND(≡), which was similar to the results with *P. putida* [6]. However, the bioconversion yield of 10-UND(≡) to PHA and the PHA content in the cells were increased maximally to 17% and 25 wt%, respectively, when the cells were grown on an equimolar mixture of 10-UND(≡) and NA. These effects were most likely due to cometabolic PHA production whereby a very poor or non-PHA producing substrate was incorporated into the PHAs, when fed to *P. oleovorans* in a mixture with other carbon substrates such as NA and OA that support PHA production in good yields [15]. Although 10-UND(=) is known as a carbon substrate that supports PHA production by *P. oleovorans*, the PHA yield from *P. oleovorans* grown solely with 10-UND(=) was significantly lower than that from cells grown solely with NA or OA [11]. Therefore, it was of interest to observe that a relatively poor carbon substrate such as 10-UND(=) would cause cometabolic PHA production, when mixed with a poor carbon substrate, 10-UND(≡).

The fermentation results of *P. oleovorans* grown in various carbon substrate mixtures are listed in Table 2. The results show that the maximal bioconversion yield of 10-UND(≡) to PHA and the PHA content in the cells were 24.8% and 42%, respectively, when 10-UND(≡) was cofed to *P. oleovorans* with the same molar fraction of 10-UND(=). These values were higher than those obtained from mixtures of 10-UND(≡) and NA as described above, clearly indicating that 10-UND(=) served as a cosubstrate and resulted in even more efficient PHA production from 10-UND(≡). Meanwhile, there was no growth when *P. oleovorans* was cultured in a medium containing 10 mM 6-heptynoic acid (6-HA(≡)) for 10 days.

It is noteworthy that the highest cell yield (2.28 g/l) and relatively lower PHA yield (0.71 g/l) were obtained when the fraction of NA was the highest as listed in Table 2. Data in Table 2 also show that the PHA yield was the highest (0.9 g/l) when the fraction of 10-UND(=) in the carbon substrate mixtures was the highest, despite the fact that the total biomass was lower than when the fraction of NA was the highest in the carbon substrate mixtures. These results suggest that *P. oleovorans* cultivated on mixtures containing NA and 10-UND(=) mainly utilized the former for growth, while it utilized the latter for the production of polyesters. These results are consistent with the results from other studies, where the fraction of repeating units from 10-UND(=) was always higher than the fraction of 10-UND(=) in carbon substrate mixtures, when the PHAs were prepared from *P. oleovorans* grown in mixtures of NA and 10-UND(=) [11]. At present, it is unclear why *P. oleovorans* would seem to prefer 10-UND(=) to NA for PHA accumulation. Meanwhile, the amount of PHA in *P. putida* cells grown in an equimolar mixture of 10-UND(=) and 10-UND(≡) was only 3% of the dry cell weight, which was significantly lower than that in *P. oleovorans*. Accordingly, these results indicate that the metabolic capability for the biosynthesis of unsaturated PHAs is significantly different between *P. oleovorans* and *P. putida*.

All the PHAs synthesized in the current study were transparent and amorphous. However, certain properties of the PHAs produced at room temperature with various molar mixtures of NA, 10-UND(=), and 10-UND(≡) were significantly different from each other; of the polyesters containing 3-hydroxyalkynoate units more than 60% were non-sticky elastomers, while the other polyesters were sticky materials. This difference may have been related to the surface free energy and surface roughness of the polymers [16]. The average molecular weight of the PHAs obtained in this study was approximately 50,000, with polydispersity indices of approximately 2.5 as determined by GPC.

Table 2. Fermentation results for *P. oleovorans* grown with various mixtures of NA, 10-UND(=), and 10-UND(≡).

Amount of carbon substrate (g/l)			Culture time (h)	DCW (g/l)	PHA content (%wt)	Fraction of C≡C units (%)[a]	PHA (g/l)	Weight of C≡C units (g/l)[b]	Yield of C≡C units (%)[c]
NA	10-UND(=)	10-UND(≡)							
	0.46	1.37	24	0.83	38.2	72.8	0.32	0.23	16.8
	0.92	0.91	28	0.98	42.0	53.0	0.41	0.22	24.8
	1.38	0.46	28	1.01	40.0	23.5	0.40	0.09	19.6
0.79	0.92	0.91	38	1.62	31.5	33.3	0.51	0.17	18.7
1.58	0.92	0.91	48	2.28	31.0	27.1	0.71	0.19	20.9
0.79	1.84	0.91	50	2.02	44.5	24.2	0.90	0.22	24.2
0.79	0.92	1.82	52	1.84	43.1	49.4	0.79	0.39	21.4

[a]Fraction of repeating units bearing C≡C bond (%).
[b]Weight of repeating units bearing C≡C bond (g/l).
[c]Yield of repeating units bearing C≡C bond (%).

In conclusion, the original goal to prepare PHAs containing C≡C bonds in substantial yields from 10-UND(≡) was successfully achieved with *P. oleovorans*, when 10-UND(=) was used as a cosubstrate. It was rather unexpected that 10-UND(=) worked as a good cosubstrate to introduce repeating units from such a poor carbon substrate as 10-UND(≡). PHAs with both C=C bonds and C≡C bonds are expected to be very useful sources of chiral (*R*)-3-hydroxyalkynoic acids as well as model compounds in investigating different reactivity and stereospecificity of reactions on C=C and C≡C bonds.

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REFERENCES

1. Chung, C. W., Y. S. Kim, Y. B. Kim, K. S. Bae, and Y. H. Rhee. 1999. Isolation of a *Pseudomonas* sp. strain exhibiting unusual behavior of poly(3-hydroxyalkanoates) biosynthesis and characterization of synthesized polyesters. *J. Microbiol. Biotechnol.* **9:** 847–853.
2. Doi, Y. and C. Abe. 1990. Biosynthesis and characterization of a new bacterial copolyester of 3-hydroxyalkanoates and 3-hydroxy-ω-chloroalkanoates. *Macromolecules* **23:** 3705–3707.
3. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. Production of unsaturated polyesters by *Pseudomonas oleovorans*. *Int. J. Biol. Macromol.* **12:** 85–91.
4. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. Bacterial polyesters containing branched poly(β-hydroxyalkanoate) units. *Int. J. Biol. Macromol.* **12:** 92–101.
5. García, B., E. R. Olivera, B. Miñambres, M. Fernández-Valverde, L. M. Cañedo, M. A. Prieto, J. L. García, M. Martínez, and J. M. Luengo. 1999. Novel biodegradable aromatic plastics from a bacterial source: Genetic and biochemical studies on a route of the phenylacetyl-CoA catabolon. *J. Biol. Chem.* **274:** 29228–29241.
6. Kim, D. Y., Y. B. Kim, and Y. H. Rhee. 1998. Bacterial poly(3-hydroxyalkanoates) bearing carbon-carbon triple bonds. *Macromolecules* **31:** 4760–4763.
7. Kim, D. Y., Y. B. Kim, and Y. H. Rhee. 2000. Evaluation of various carbon substrates for the biosynthesis of polyhydroxyalkanoates bearing functional groups by *Pseudomonas putida*. *Int. J. Biol. Macromol.* **28:** 23–29.
8. Kim, D. Y., S. B. Jung, G. G. Choi, Y. B. Kim, and Y. H. Rhee. 2001. Biosynthesis of polyhydroxyalkanoate copolyester containing cyclohexyl groups by *Pseudomonas oleovorans*. *Int. J. Biol. Macromol.* **29:** 145–150.
9. Kim, S. N., S. C. Shim, D. Y. Kim, Y. H. Rhee, and Y. B. Kim. 2001. Photochemical crosslinking and enzymatic degradation of poly(3-hydroxyalkanoate)s for micropatterning in photolithography. *Macromol. Rapid Commun.* **22:** 1066–1071.
10. Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1992. Poly(β-hydroxyalkanoate) copolymers containing brominated repeating units produced by *Pseudomonas oleovorans*. *Macromolecules* **25:** 1852–1857.
11. Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1995. Poly-3-hydroxyalkanoates containing unsaturated repeating units produced by *Pseudomonas oleovorans*. *J. Polym. Sci.* (*Part A: polymer chemistry*) **33:** 1367–1374.
12. Kim, Y. B., D. Y. Kim, and Y. H. Rhee. 1999. PHAs produced by *Pseudomonas putida* and *Pseudomonas oleovorans* grown with *n*-alkanoic acids containing aromatic groups. *Macromolecules* **32:** 6058–6064.
13. Lee, S. Y., Y. Lee, and F. Wang. 1999. Chiral compounds from bacterial polyesters: Sugars to plastics to fine chemicals. *Biotechnol. Bioeng.* **65:** 363–368.
14. Legeveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt. 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. *Appl. Environ. Microbiol.* **54:** 2924–2932.
15. Lenz, R. W., Y. B. Kim, and R. C. Fuller. 1992. Production of unusual bacterial polyesters by *Pseudomonas oleovorans* through cometabolism. *FEMS Microbiol. Rev.* **103:** 207–214.
16. Russell, T. P. and H. C. Kim. 1999. Tack - a sticky subject. *Science* **285:** 1219–1220.
17. Song, J. J. and S. C. Yoon. 1996. Biosynthesis of novel aromatic copolyesters from insoluble 11-phenoxyundecanoic acid by *Pseudomonas putida* BM01. *Appl. Environ. Microbiol.* **62:** 536–544.
18. Song, J. J., M. H. Choi, S. C. Yoon, and N. E. Huh. 2001. Cometabolism of ω-phenylalkanoic acids with butyric acid for efficient production of aromatic polyesters in *Pseudomonas putida* BM01. *J. Microbiol. Biotechnol.* **11:** 435–442.