Biosynthesis of Poly(3-hydroxyalkanoate)s with Terminal Alkene Groups in the Side Chains by *Chromobacterium* sp.

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Chromobacterium sp. isolated from a river soil produced the poly(3-hydroxyalkanoate)s [P(3HA)s] with unsaturated groups at the terminal positions of the β-substituents when 10-undecenoic acid (UDEA) was fed as the carbon sources.

Microbial poly(hydroxyalkanoate)s (PHAs) are a biodegradable thermoplastic with a wide range of physical properties.[1,2] A number of microorganisms biosynthesized the isotactic homopolymers and the copolymers of (R)-3-hydroxyalkanoic acids(3HA) with three to fourteen carbon atoms as an intracellular strage material of carbon and energy.[3,4] More than 90 different hydroxyalkanoic acids are now known as constituents of bacterial PHA.[5] Of the microorganisms, capable of producing PHAs, *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) and *Pseudomonas oleovorans* have been investigated most extensively.[6,7] *P. oleovorans* and other pseudomonands belonging to the *r*RNA homology I[8] especially produce different PHAs from a variety of carbon substrates, including *n*-alkanes, alkanoic acids, alkenoic acid and halo-alkanoic acids, etc. The productions of PHAs containing functional groups such as branched alkyl,[9] halogen,[10-12] phenyl,[13,14] olefin,[7,15,16] nitryl,[17] and ester groups[18], have been reported. Since these microbial polyesters are functional biodegradable thermoplastics, they have attracted much attention as new environmentally compatible materials.[19,20] Espetially, PHAs containing olefin units have potential for different applications, because these PHAs could be converted into other more useful functional groups such as alcohol and carboxylic acid, or crosslinked easily to improve their mechanical properties. *Pseudomonas oleovorans*[7,15,16] and *Rhodospirillum rubrum*[21] have been to date shown to produce PHAs containing terminal alkene groups in the side chains when grown on the substrates with terminal alkenes as carbon sources. The PHAs produced by *R. rubrum* on 4-pentenoic acid contained significant amounts of saturated units, presumably by hydrogeneration of the unsaturated carbon sources in the cells. In constrast, the PHAs produced by *P. oleovorans* grown on ω-alkenoic acids such as 7-octenoic acid or 10-undecenoic acid (UDEA) contained up to 98 mol% of alkene units in the repeating units, but the biomass and polyester yields were as low as 0.6 g dm^{-3} and 0.2 g dm^{-3}, respectively.

In the present study, we report the biosynthesis of PHAs with fully terminal alkene groups in all side chains from UDEA as sole carbon source by a new *Chromobacterium* sp. strain isolated from river soil. In *Chromobacterium* genus, *violaceum* strain[22] has been to date reported to produce the PHAs with 3-hydroxybutyrate(3HB) and 3-hydroxyvalerate(3HV) units in these repeating units, but no production of PHAs containing unsaturated units has been reported. We have reported that a new *Chromobacterium* sp. isolated from a soil at Hottate river (Yonezawa city) produced effectively P(3HB) from different plant oils, and moreover produced simultaneously a copolymer with 4-hydroxybutyrate(4HB) units and P(3HA) containing medium-chain-length saturated and unsaturated 3HA units from 4-hydroxybutyric acid.[23,24]

The microbial polyester synthesis from UDEA was carried out by two-stage cultivation. *Chromobacterium* strain was first grown in the nutrient-rich medium containing yeast extract, polypeptone, meat extract and ammonium sulfate at 30 ℃ for 24 h. The cells were harvested by centrifugation, and transferred into nitrogen-free media containing UDEA. The cells were incubated for prescribed time at 30 ℃, harvested, washed and lyophilized. The polyesters were extracted from the lyophilized cells with hot chloroform. Chloroform was evaporated *in vacuo* and residual polymers were dissolved in hexane and hexane-insoluble product was filtrated. The filtrate was condensed under reduced pressure , and to the residual viscous oil the methanol was added and methanol-insoluble polymer was washed thoroughly with methanol.

Table 1 shows the results of the PHA production and the PHA compositions in *Chromobacterium* sp. from UDEA alone. UDEA is prepared by pyrolysis of castor oil, and hence renewable and inexpensive resource. The polyester accumulation was observed when the cultivation medium contains 0.3-1.0 wt% of UDEA. When cultivated on medium containing 1%(w/v) UDEA for 48 h, the cell dry matter and PHA weights were 3.02 and 0.72 g dm^{-3}, respectively. These values are four times as higer as

Table 1. Production and Compositions of PHAs from UDEA by *Chromobacterium* sp. for 48 h at 30 °C

UDEA conc.	Cell dry weight	PHA weight	PHA content[a]	PHA composition[b] / mol%		
g dm^{-3}	g dm^{-3}	g dm^{-3}	wt%	3HHp(=)	3HN(=)	3HUD(=)
3	2.64	0.11	4.0	3	67	30
5	2.81	0.15	5.5	6	70	24
10	3.02	0.72	24.0	7	73	20

[a]PHA content in cell dry weight. [b]Determined by ^{1}H-NMR and GC: 3HHp(=); 3-hydroxy-6-heptenoate, 3HN(=); 3-hydroxy-8-nonenoate, 3HUD(=); 3-hydroxy-10-undecenoate.

those of PHA produced in *P. oleovorans* fom UDEA as a sole carbon source. The compositions of PHAs were determined from ^{1}H NMR, ^{13}C NMR and gas chromatography (GC) analyses,[7] The ^{1}H NMR spectrum of PHA is shown in Figure1. The methine proton in the 3 position of PHA was obserbed at 5.4 ppm, which was same chemical shift in the normal region for this groups. The peaks for the olefin protons in the side chain of PHA appeared at 5.2 and 5.4 ppm. No peaks for methyl protons were obserbed. The integration of the ^{1}H NMR spectra indicated that the PHA from UDEA contained fully unsaturated repeating units in this side chains. ^{13}C NMR spectrum of PHA is shown also in Figure 1, together with the chemical-shift assignments for each carbon resonances. The chemical-shifts of all carbon resonances could be

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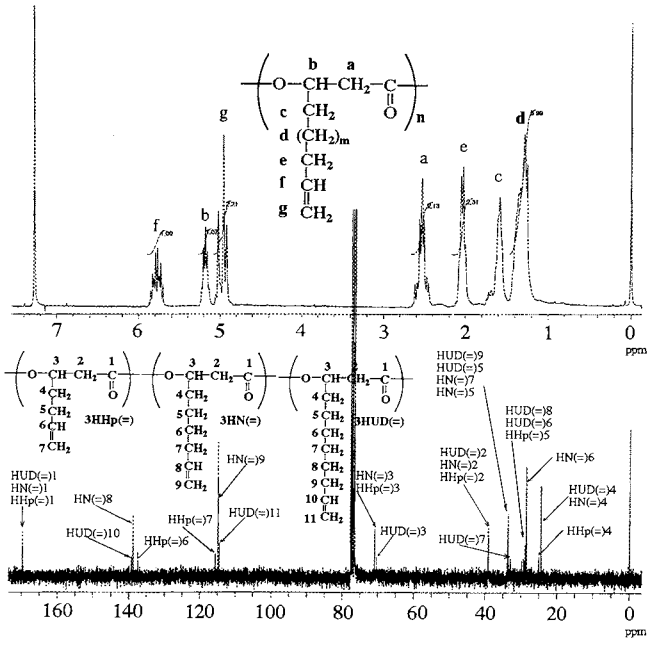

Figure 1. ^{1}H-NMR and ^{13}C-NMR spectra of PHA from 10-undecenoic acid by *Chromobacterium* sp. in $CDCl_3$.

assigned by reference to the data of the literature.[16] The six split peaks in the rigion between 115 and 140 ppm show clearly the presence of the terminal alkene groups in the side chains of PHA. The results of GC analysis of the methanolyzed samples of PHA are shown in Table1. The PHAs produced by *Chromobacterium* sp. on UDEA contained primarily the 3-hydroxy-8-nonenoate repeating unit in addition to 3-hydroxy-10-undecenoate and 3-hydroxy-6-heptenoate as minor repeating unit. This PHA was very viscous polymer at room temperature, and a single glass-transition temperature was observed at -50 °C. The number average molecular weight($\overline{M}_n$) and polydispersity index ($\overline{M}_w/\overline{M}_n$) of the PHA estimated by gel permeation chromatography (using polystyrene standards) were 41000 and 2.6 respectively.

In *Chromobacterium* sp the incorporation of 3-hydroxyacids with terminal olefin possessing two or four fewer carbon atoms than UDEA may involve the removal of C_2 units by β-oxidation cycle, as demonstrated in PHA biosynthesis by *Pseudomonas oleovorans*.[7]

Futher studies are being carried out to determine the effcts of varying the culture conditions on the productivities as well as the properties of the PHA with terminal alkenes in the side chains.

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