Production of Polyester Blend of Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and Poly(3-hydroxyalkanoate) with Saturated and Unsaturated Monomers from 4-Hydroxybutyric Acid by *Chromobacterium* sp.

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Chromobacterium sp. isolated from a river soil produced a polyester blend of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)(P(3HB-*co*-4HB)) and poly(3-hydroxyalkanoate) (P(3HA)) consisting of both saturated 3-hydroxyalkanoic acid(3HA) units of even carbon number from C4 to C14 and unsaturated 3HA units of carbon number C12 and C14, when 4-hydroxybutyric acid(4HBA) was fed as the sole carbon sources.

Poly(hydroxyalkanoates)(PHAs) are bacterial storage compounds, which are produced and intracellularly deposited as granules in many bacteria in the presence of excess carbon source under restricted growth conditions.[1] More than 90 differnt constituents of PHAs have been identified to date as different hydroxyalkanoic acids with 3-14 carbon atoms.[2] Since these microbial polyesters are biodegradable thermoplastics and /or elastomers, they have attracted much attention as a new environmentally compatible materials.[3,4] The PHA producing bacteria can be broadly divided into two groups. One group of bacteria including *Ralstonia eutropha*(reclassified from *Alcaligenes eutrophus*) produces short-chain PHAs with C3-C5 monomer units, while the other group including *Pseudomonas oleovolans* produces medium-chain PHAs with C6-C14 monomer units.[5,6]

In recent studies, a few bacteria have been found to produce polyesters consisting of 3HB and medium-chain-length 3HA units. *Rhodospirillum rubrum*,[7] *Rhodocyclus gelatinosus*[8] and *Rhodococcus ruber*[9] produced terpolymers consisting of 3HA units of C4, C5 and C6. *Aeromonas caviae*[10] produced a random copolymer of 3HA units of C4 and C6. Some *Pseudomonas* strains and other fluorescent Pseudomomonads were found to accumulate copolyesters containing 3HA units of C4 to C16.[11,12] In genral, bacterial copolyesters of hydroxyalkanoic acids have a narrow distribution of copolymer composition.[13,14] However, the PHAs produced by a *Psuedomonas* strain have been shown to be a mixture of P(3HB) homopolymer and random copolymer of 3HA units of C4 to C12 when grown on alkanoic acids.[15]

In this paper, we report that *Chromobacterium* strain produces a blend of P(3HB-co-4HB) and copolyester consisting of the saturated 3HA units of even carbon numbers C4 to C14 in addition to two unsaturated 3HA units (3-hydroxy-5-*cis*-dodecenate(3H5DD) and 3-hydroxy-7-*cis*-tetradecenoate (3H7TD)) when cultivated on 4-hydroxybutyric acid(4HBA) as the sole carbon source under nitrogen-free conditions. To our knowlege, this *Chromobacterium* sp. is the first strain to produce simultaneously P(3HB-*co*-4HB) and P(3HA) containing 3HB and medium-chain-length 3HA units from 4HBA.

A new *Chromobacterium* strain isolated from a soil at Hottate river (Yonezawa city), possessed purple pigment, and was a Gram-negative, aerobic and motile rod. Further, this strain revealed the ability reducing nitrate, oxidase- and catalase-positive, and was confirmed to be fermentative species by Hugh-Leifson method.[16] The identification tests of the isolated strain by using API 20NE test kit [17] exhibited some comparable behaviors to *Chromobacterium violaceum*, but this strain was different from it in several phenotypic properties examined. The microbial polyester synthesis from 4HBA 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 4HBA. 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 separated into hexane-soluble and hexane-insoluble polymers. Each polymer was washed thoroughly with methanol.

Table 1 shows the results of the polyester production in *Chromobacterium* sp. from 4HBA. The polyester accumulation is observed when the cultivation medium contains an excess of 4HBA (>10 gdm^{-3}). When cultivated on medium containing 2%(w/v) 4HBA, the contents of polyesters in cell dry weights were 14.1 wt% and the fractions of hexane-soluble and insoluble polymers were 48 wt% and 52 wt%, respectively. The

Table 1. Biosynthesis of polyesters from 4-hydroxybutyric acid (4HBA) for 48 h at pH 7.0 and 30 °C by *Chromobacterium* sp.

Run	4HBA conc.	Cell dry weight	Polyester content[a]	Polyester fraction / wt%	
	g dm^{-3}	g dm^{-3}	wt%	hexane-insoluble	hexane-soluble
1	5	3.71	1.4	100	0
2	10	4.90	3.1	69	31
3	15	5.28	7.2	61	39
4	20	5.80	14.1	52	48
5	30	5.82	13.0	86	14

[a] Polyester content in cell dry weight.

Table 2. Compositions of hexane-soluble and insoluble polymers produced from 4HBA by *Chromobacterium* sp.

Polymer of Run 4	Polyester composition [a] / mol%								
	3HB	4HB	3HH	3HO	3HD	3HDD	3H5DD	3HTD	3H7TD
hexane-soluble	1.5	0	3.5	21.3	63.9	2.3	5.4	1.0	0.9
hexane-insoluble	81.5	19.5	0	0	0	0	0	0	0

[a] Determined by ^{1}H-NMR and GC: 3HB; 3-hydroxybutyrate, 4HB; 4-hydroxybutyrate, 3HH; 3-hydroxyhexanoate, 3HO; 3-hydroxyoctanoate, 3HD; 3-hydroxydecanoate, 3HDD; 3-hydroxydodecanoate, 3H5DD; 3-hydroxy-5-*cis*-dodecenoate, 3HTD; 3-hydroxytetradecanoate, 3H7TD; 3-hydroxy-7-*cis*-tetradecenoate.

compositions of hexane-soluble and insoluble polymers were determined from ^{1}H-NMR, ^{13}C-NMR and gas chromatography (GC) analyses,[15] and the results showed in Table 2. The hexane-insoluble polymer are composed of two monomeric units of 3HB and 4HB by comparing with reference data.[18] While, the repeat-unit compositions for hexane-soluble polymer were similar to those analyzed by Huijiberts *et al.*[19] and Kato *et al.*[20] for the PHA isolated from *Pseudomonas* strains grown on sugars. Figure 1 shows the 67.5 MHz ^{13}C-NMR spectrum of hexane-soluble polymer, together with the chemical-shift assignments for each carbon resonances and an expanded spectrum of carbonyl resonances. The chemical-shifts of all carbon resonances could be assigned by reference to the data of the literature.[20] The four peaks in the rigion between 122 and 134 ppm clearly provided evidence for the presence of the two unsaturated 3HA monomer units: 3-hydroxy-5-*cis*- dodecenate(3H5DD) and 3-hydroxy-7-*cis*-tetradecenoate(3H7TD). The carbonyl carbon resonances (169-169.6 ppm) are resolved into three groups of peaks, arising from different diad sequences of connecting 3HB and other 3HA units of carbon numbers C6 to C14. Thus it suggested that the sequence distribution of the 3HB and medium-chain-length 3HA units of the hexane-soluble polymer is statistically random. The major constituent of hexane-soluble polymer was 3-hydroxydecanoate (3HD) by GC analysis (Table 2). The number average molecular weight ($\overline{M}_n$), the melting temperature(T_m) and the glass tansition temperature(T_g) of hexane-insoluble polymer (P(3HB-*co*-20 mol%4HB)) are *ca.* 2.1 X10^5, 160 °C and -5 °C , while, those of hexane-soluble polymer (Run 4) are *ca.* 8 X10^4, 48 °C and -43 °C, respectively.

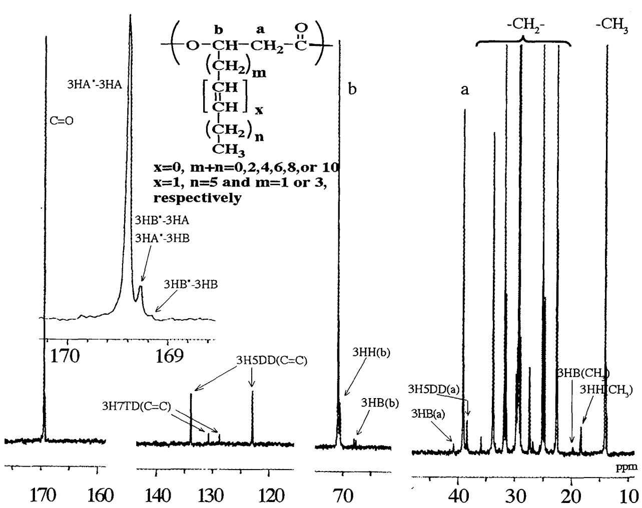

Figure 1. ^{13}C-NMR spectrum of hexane-soluble polymer in $CDCl_3$. Abbreviations of 3HB, 3HH, 3H5DD and 3H7TD see footenote of Table 2.

Recently, the pathway of the biosynthesis of P(3HA) containing both saturated and unsaturated 3HA units from sugar by *Pseudomonas* sp. has been related to *de novo* fatty acid biosynthesis.[19,20] Therefore, the pathway of the biosynthesis of P(3HA) with medium-chain-length 3HA units from 4HBA by *Chromobacterium* sp. suggests also possible linkage of *de novo* fatty acid biosynthesis. Further studies of biosynthesis mechanism on PHA production from 4HBA by *Chromobacterium* sp. are in progress.

In conclusion, it has been demonstrated that a new *Chromobacterium* sp. can produce a blend of P(3HB-co-4HB) and PHA containing the saturated 3HA units of even carbon numbers C4 to C14 in addition to two unsaturated 3HA units of C12 and C14 when cultivated on 4HBA

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