Noda Institute for Scientific Research GRANT
2011 Young Investigator Research Grant

Genetic engineering for copolymerized bio-polyester production from methanol

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Research aims

Polyhydroxyalkanoate (PHA) is an intracellular biopolyester, which is synthesized by various microorganisms as the energy storage compound. This biopolyester can be produced from sugar and plant oil derived by biomass; furthermore, it has biodegradability and biocompatibility. Therefore, PHA is attracting interest as a substitute for petroleum-derived plastics. The most representative PHA is poly(3-hydroxybutyrate)[P(3HB)], which is the homopolymer composed of only C_4 monomer unit. However, P(3HB) is not suitable for practical use because its physical property is stiff and brittle. On the other hand, copolyesters copolymerized with medium-chain-length monomer (C_6–C_{10}) have flexible properties. In this study, genetic engineering of microorganisms was performed for efficient production of PHA copolymer composed of C_4 and C_6 monomer units from methanol, which is inexpensive and sustainable feedstock. For this purpose, three kinds of methylotrophic bacteria (methylotrophs), capable of growth on methanol as a sole carbon source, and a non-methylotrophic representative PHA producer were used as the host strains.

Methods

General genetic manipulation was performed using *Escherichia coli* DH5α and S17-1. Three methylotrophs, *Methylobacterium extorquens* AM1, *Paracoccus denitrificans*, and *Methylobacillus flagellatus* and hydrogen oxidizing bacterium *Ralstonia eutropha* (non-methylotroph) were used as the host strains. The target genes were amplified by PCR and then ligated with modified pCM80[1] or pK18mobsacB for gene expression or homologous recombination, respectively. The accumulated PHAs were analyzed as follows. The cells were grown in nitrogen-limited medium containing 0.5% or 1% (v/v) methanol. After the cultivation, the cells were harvested, followed by lyophilized. Cellular PHA content and composition were determined by GC and GC-MS after methanolysis of the dried cells in the presence of 15% sulfuric acid. Furthermore, the extracted PHAs were analyzed by GPC and NMR.

Results

1. *Genetic engineering of M. extorquens and P. denitrificans.*

First of all, we have reanalyzed PHAs produced by *M. extorquens* AM1 wild-type strain and a previously constructed mutant strain, $AM1C_{Ac}$, in which its PHA synthase gene was replaced by a gene of the *Aeromonas caviae* PHA synthase ($phaC_{Ac}$) with a broad specificity. The PHA produced by *M. extorquens* AM1 from methanol was previously thought to be P(3HB). Surprisingly, our results revealed that *M. extorquens* AM1 synthesized poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] copolymer copolymerized with C_5 monomer unit. On the other hand, the mutant strain $AM1C_{Ac}$ accumulated poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-co-3-hydroxyhexanoate) [P(3HB-*co*-3HV-co-3HHx)] terpolymer composed of C_4, C_5 and C_6 monomer units (Table 1). Next, the genes encoding enzymes for supplying the precursor of second monomer focusing on CoA intermediate on the ethylmalonyl-CoA pathway, which is for regeneration of glyoxylate in this bacterium[2], were introduced in *M. extorquens* $AM1C_{Ac}$. As a result, when the gene encoding crotonyl-CoA carboxylase/reductase was expressed, C_6 monomer composition was increased 2 times compared with the parent strain, though C_6 monomer composition was still low. We also succeeded in increasing PHA production and C_5 monomer composition by expression of the *phaA* and *phaB* genes encoding monomer supplying enzymes (Table 1).

It was reported that *P. denitrificans* accumulates P(3HB) from methanol[3]. Its PHA polymerase gene was replaced with the $phaC_{Ac}$ gene or its mutant $phaC_{NSDG}$ gene by homologous recombination. However, unfortunately, these transformants accumulated only P(3HB) from methanol like the parent strain.

2. *Conferring PHA synthesis ability on M. flagellatus*

M. flagellatus is an obligate methylotroph, which can grow on methanol very efficiently, but is unable to synthesize PHA. In this methylotroph, we expressed the *phaA* and *phaB* genes from *R. eutropha* and the $phaC_{NSDG}$ genes under the control of its own methanol dehydrogenase promoter. As the result of cultivation, the transformant accumulated 7 wt% P(3HB), though its growth rate was lower than that of the parent strain.

Table 1. Biosynthesis of PHA from methanol by *M. extorquens* AM1 and recombinant strains.

Strain	Cultivation time[a] (h)	DCW[b] (g/l)	RCW[c] (g/l)	PHA content (wt%)	Composition (mol%)		
					3HB (C_4)	3HV (C_5)	3HHx (C_6)
AM1	72	0.699	0.642	8.38	95.7	3.92	0
$AM1C_{Ac}$	72	0.874	0.580	32.6	99.2	0.710	0.280
$AM1C_{Ac}$/pCM80-ccr	96	0.720	0.539	25.1	99.5	0.528	0.528
$AM1C_{Ac}$/pCM80Km-phaAB	72	0.416	0.241	43.6	96.6	3.23	0.201

[a] Cells were cultured on the medium containing 0.5% methanol.
[b] Dry cell weight.
[c] Residual cell weight.

3. *Conferring methanol utilization ability on R. eutropha*

R. eutropha accumulates more than 80 wt% PHA when grown on sugar and plant oil, but is unable to grow on methanol. Analyses of the genome sequence revealed that this bacterium has homologs of enzymes involved in methanol oxidation. Therefore, we overexpressed them in *R. eutropha*. Characterization of the transformants is in progress.

Conclusion

In this study, genetic engineering techniques were applied to strains of methylotroph and non-methylotroph for efficient PHA copolymer production from methanol. We succeeded in constructing a strain of methylotroph, which can efficiently synthesize PHA copolymer composed of not only short-chain-length monomers but also C_6 monomer unit. We also succeeded in conferring PHA production ability on a strain of methylotroph, which is originally unable to synthesize PHA. However, both composition of C_6 monomer and PHA productivity are still low. Further strain improvement through genetic engineering is required.

References

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