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Biosynthesis of (*R*)-3-Hydroxyalkanoic Acids by Metabolically Engineered *Escherichia coli*

Si Jae Park,[†,1] Sang Yup Lee,[*,1,2] and Young Lee[1,3]

[1]Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical & Biomolecular Engineering, BioProcess Engineering Research Center and [2]Department of BioSystems and Bioinformatics Research Center, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea, E-mail: leesy@kaist.ac.kr; and [3]ChiroBio Inc., #6102-6103, KAIST Alumni Venture Hall, 400 Guseong-dong, Yuseong-gu, Daejeon 305-338, Republic of Korea

Abstract

An efficient system for the production of (*R*)-hydroxyalkanoic acids (RHAs) was developed in natural polyhydroxyalkanoate (PHA)-producing bacteria and recombinant *Escherichia coli*. Acidic alcoholysis of purified PHA and in vivo depolymerization of PHA accumulated in the cells allowed the production of RHAs. In recombinant *E. coli*, RHA production was achieved by removing CoA from (*R*)-3-hydroxyacyl-CoA and by in vivo depolymerization of PHA. When the recombinant *E. coli* harboring the *Ralstonia eutropha* PHA biosynthesis genes and the depolymerase gene was cultured in a complex or a chemically defined medium containing glucose, (*R*)-3-hydroxybutyric acid (R3HB) was produced as monomers and dimers. R3HB dimers could be efficiently converted to monomers by mild alkaline heat treatment. A stable recombinant *E. coli* strain in which the *R. eutropha* PHA biosynthesis genes were integrated into the chromosome disrupting the *pta* gene was constructed and examined for the production of R3HB. When the *R. eutropha* intracellular depolymerase gene was expressed by using a stable plasmid containing the *hok/sok* locus of plasmid R1, R3HB could be efficiently produced.

Index Entries: (*R*)-Hydroxyalkanoic acids; polyhydroxyalkanoate; *Escherichia coli*; poly-(*R*)-3-hydroxybutyrate; (*R*)-3-hydroxybutyric acid.

[†]Present address: LG Chem, Ltd., 104-1, Moonji-dong, Yuseong-gu, Daejeon, 305-380 Republic of Korea.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Polyhydroxyalkanoates (PHAs) are a group of completely biodegradable polyesters that are synthesized in many bacteria under unfavorable growth conditions in the presence of excess carbon source *(1–4)*. More than 150 types of alkanoic acids hydroxylated at the 3-, 4-, 5-, or 6-position, all in (*R*)-configuration if they possess an asymmetric center at the carbon position linked to the hydroxyl group, can be incorporated into PHAs *(5)*. These (*R*)-hydroxyalkanoic acids (RHAs) contain two functional groups that can easily be modified to produce many chiral compounds, especially fine chemicals such as antibiotics, vitamins, perfumes, and pheromones *(6–8)*. For example, (*R*)-3-hydroxybutyric acid (R3HB) is an important precursor of 4-acetoxyazetidinone, which is used for the synthesis of the antibiotics-carbapenem, which has a market worth of almost $1 billion. Methods for producing RHAs by chemical digestion of PHAs have been reported *(8–11)*. However, large amounts of organic solvents were used, and the production efficiency was rather low owing to complex processes.

Poly-(*R*)-3-hydroxybutyrate (PHB) is the most ubiquitous member of PHAs. The metabolism for the synthesis and degradation of PHB plays an important role in many bacteria for the reservation and reutilization of excess carbon/energy source and reducing power *(1)*. In short-chain-length PHA-producing bacteria, PHB is synthesized from acetyl-CoA by three sequential enzymatic reactions catalyzed by β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase *(12–14)* (Fig. 1). PHB is known to be depolymerized to R3HB by intracellular PHA depolymerase and oligomer hydrolase *(1,2)*. R3HB is further converted to acetoacetate by R3HB dehydrogenase. By employing this cyclic nature of PHB synthesis and degradation, we have recently demonstrated that R3HB could be efficiently produced in the natural PHA-producing bacteria *Alcaligenes latus* by lowering the fermentation pH, under which high activity of intracellular PHA depolymerase was observed, but no activity of R3HB dehydrogenase was found *(15)*.

Recombinant *Escherichia coli* strains harboring the heterologous PHA biosynthesis genes have been shown to be suitable for the high-level production of PHAs. In addition, several engineered metabolic pathways for the synthesis of various RHAs have recently been established in recombinant *E. coli (16–18)*.

In this article, we review the development of processes for the production of RHAs in natural PHA-producing bacteria and metabolically engineered *E. coli* strains.

Bioconversion

Conventional methods for the production of R3HB by bioconversion include chiral oxidation of 1,3-butanediol by bacteria *(19)*, microbial hydroxylation of butyric acid, and microbial or enzymatic reduction of alkyl-3-ketobutyrate *(20,21)*. However, all of these methods have the problem of low productivity and are therefore difficult to industrialize.

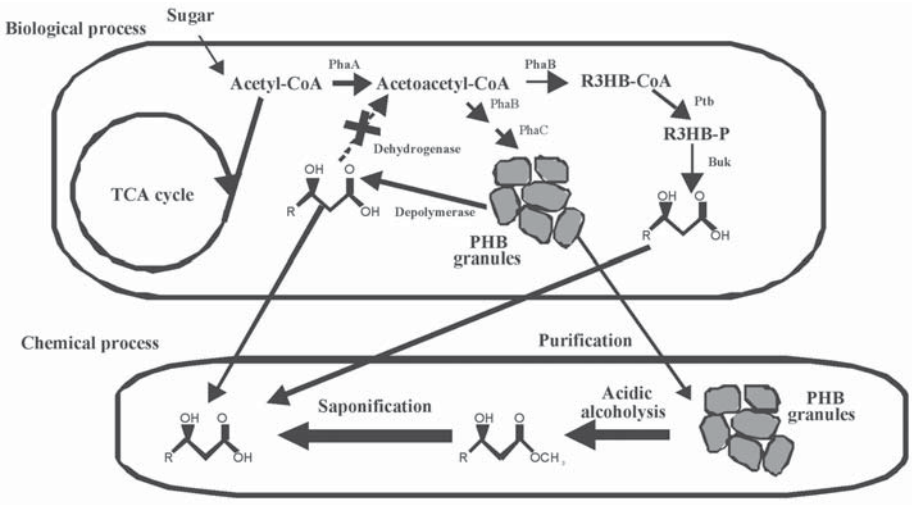

Fig.1. Biologic and chemical processes for production of R3HB in natural and recombinant bacteria. PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; Ptb, phosphotransbutyrylase; Buk, butyratekinase; TCA, tricarboxylic acid.

Chemical Process of PHA Degradation

Chemical processes for the production of RHAs and RHA alkyl esters are composed of two steps *(9,22)* (Fig. 1). First, PHA is synthesized in natural or recombinant bacteria and then purified. Cells containing PHA can also be used. Second, purified PHA (or cells containing PHA) is depolymerized by acidic alcoholysis using sulfuric acid or hydrochloric acid as an acidic catalyst to produce RHA alkylesters. Subsequent saponification of the purified RHA alkylesters yields corresponding RHAs.

PHB produced by recombinant *E. coli* harboring the *R. eutropha* PHA biosynthesis genes was subjected to acidic alcoholysis, resulting in the production of R3HB methylester *(9)*. One of the disadvantages of acidic alcoholysis is that the purity of PHA should be high enough to support good yield, and, therefore, the production cost is rather high owing to the expensive polymer purification procedure. Recombinant *E. coli* seems to be suitable for the development of processes for the economical production of R3HB because it allows production of a large amount of PHB and economical purification of PHB by a simple NaOH digestion method *(23)*.

Medium-chain-length (MCL) (*R*)-3-hydroxyalkanoic acid (R3HA) methylesters having carbon numbers of 6–12 were produced by acidic methanolysis of MCL-PHA purified from *Pseudomonas putida (22)*. Because MCL-PHA is composed of several monomers, the produced R3HA methylester mixtures were further seperated using distillation. Distillation yield decreased with increasing carbon numbers of MCL-R3HA methylesters, resulting in 99.9, 99.8, 88.4, and 56.8% for 3-hydroxyhexanoic, 3-hydroxyoctanoic, 3-hydroxydecanoic, and 3-hydroxydodecanoic acid

methylester, respectively. MCL-R3HA methylesters could be easily converted into R3HAs by saponification *(22)*.

Biologic Process of PHA Degradation

Construction of Pathways for Production of RHAs Without PHA Synthesis

When two of the PHA biosynthesis genes coding for β-ketothiolase and reductase are expressed without PHA synthase gene, (*R*)-hydroxyacyl-CoAs (R3HA-CoAs) should be accumulated in the cytoplasm. The key challenge of this process toward R3HA production is how to efficiently remove CoA from R3HA-CoA. Recently, it was found that *E. coli* possesses unknown enzymes that are able to remove the CoA moiety from R3HA-CoA *(16,17)*. The pathways for the production of R3HB and MCL-R3HA were constructed in recombinant *E. coli*. When the recombinant *E. coli* DH5α harboring the *R. eutropha phaAB* genes was cultured on glucose, R3HB was produced and excreted into the medium up to 0.66 g/L. In addition, the expression of the *P. putida phaG* gene encoding 3-hydroxydecanoyl-ACP:CoA transacylase in recombinant *E. coli* DH5α resulted in production of the mixtures of 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid up to 0.19 g/L *(16)*.

A more efficient pathway for the production of R3HB was constructed by the coexpression of the *R. eutropha phaAB* genes, and the phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) genes in *E. coli* (Fig. 1). CoA was efficiently removed from R3HB-CoA by conversion of R3HB-CoA to R3HB-P, and finally to R3HB by butyrate kinase. Up to 1.4 g/L of R3HB was produced by culturing this recombinant *E. coli* DH5α on glucose. In addition, fed-batch culture of this recombinant *E. coli* yielded 12 g/L of 3HB in 48 h *(17)*.

Construction of Pathways for Production of RHAs with PHA Synthesis

Using Natural PHA-Producing Bacteria

The key concept consists of PHA biosynthesis and subsequent depolymerization of produced PHA in cells; therefore, it can be called an in vivo PHA depolymerization process *(15)*. RHAs produced by the depolymerization of PHAs can be excreted into the medium when the further intracellular metabolism of RHAs is inhibited. Because all the PHA-producing bacteria also possess PHA degradation pathways, in vivo depolymerization of PHA can be achieved by natural PHA-producing bacteria when the proper conditions are met.

We have recently reported the development of a process for the in vivo depolymerization of PHA in several natural PHA producing bacteria including *A. latus*, *R. eutropha*, and pseudomonads *(15)*. Metabolic pathways involved in the synthesis and degradation of PHB are shown in Fig. 1.

As seen from the metabolic pathways, it is important to maintain the highest activity of depolymerase with the lowest or no R3HB dehydrogenase activity for the successful production of R3HB. Because PHA is first accumulated and subsequently depolymerized into monomers, the initial amount of accumulated PHA is important to achieve efficient monomer production. Different concentrations (10.1 g/L and 135.6 g/L) of *A. latus* cells containing PHB were examined for the production of R3HB *(15)*. When these cells were incubated at pH 4.0, which supports highest depolymerase and lowest R3HB dehydrogenase activities, 8.7 and 117.8 g/L of R3HB, respectively, were obtained. Other RHAs such as (*R*)-3-hydroxyvaleric acid (R3HV) and MCL-R3HAs could also be produced by in vivo depolymerization of corresponding PHAs accumulated in *R. eutropha* and pseudomonads *(15)*. These results suggest that more than 150 different types of R3HA can be produced by accumulating and depolymerizing PHA in cells having PHA metabolism using the strategy described *(5)*.

Using Metabolically Engineered *E. coli*

It has previously been demonstrated that recombinant *E. coli* strains harboring the *R. eutropha* PHA biosynthesis genes *(12–14)* are able to accumulate a large amount of PHB, up to 90% of dry cell weight *(24)*. Recently, the intracellular PHB depolymerase gene was cloned from *R. eutropha (25)*. It was therefore reasoned that R3HB and other RHAs may be efficiently produced by establishing a heterologous PHA biosynthesis and degradation pathway in *E. coli*. It is advantageous that *E. coli* does not possess a metabolic pathway consuming R3HB. For the construction of a R3HB production system employing recombinant *E. coli*, a plasmid containing the *R. eutropha* PHA biosynthesis genes and the PHA depolymerase gene was constructed and transformed into *E. coli (18)*. When this metabolically engineered *E. coli* was cultured in complex or chemically defined medium containing glucose, R3HB was produced as monomers and dimers. R3HB dimers could be efficiently converted to monomers by mild alkaline heat treatment. Additionally, R3HV could be produced by culturing recombinant *E. coli* on glucose and propionic acid as carbon sources *(18)*.

During the batch culture of recombinant *E. coli* for the production of R3HB, it was found that the concentration of R3HB dimers increased at the expense of R3HB monomer concentration (Table 1; *[18]*). This result suggests that some of the R3HB monomers excreted are esterified to form R3HB dimers. Generally, the carboxylic acid group of R3HB can readily be esterified with alcohol groups to form esters *(26,27)*. Since R3HB molecules possess both alcohol and carboxylic acid functional groups, the produced R3HB monomers can serve as reactants for reversible intermolecular esterification to form R3HB dimers. When R3HB was excreted mainly as monomers by the depolymerization of PHB during the exponential growth phase, the ratio of R3HB monomer to its dimer was high enough to proceed with the esterification reaction. Nonetheless, R3HB could be successfully obtained by mild alkaline heat treatment *(18)*.

Table 1
Summary of Batch Cultivation of Recombinant *E. coli* XL1-Blue for the Production of R3HB[a]

Plasmid	Cultivation time (h)	Cell mass (g/L)	Concentration (g/L)			Monomer yield (%)
			PHB	R3HB[b]	R3HB[c]	
Single plasmid[d]	30	1.29	1.29	2.38	9.60	48.1
Two plasmids[e]	30	0.46	ND[f]	1.67	9.89	49.5

[a] Data are taken from ref. *18*.
[b] R3HB concentration produced in monomeric form.
[c] R3HB concentration obtained after alkaline heat treatment.
[d] Plasmid pSYL105Red containing *R. eutropha* PHA biosynthesis genes and depolymerase gene was used.
[e] Plasmids p5184 containing *R. eutropha* PHA biosynthesis genes and pUC19Red containing *R. eutropha* depolymerase gene were used.
[f] Not detected.

To solve the problem that some PHB was left over in recombinant *E. coli*, the two-plasmid system, in which depolymerase gene was cloned into a high-copy-number plasmid, pUC19, and the PHA biosynthesis genes into a low-copy-number plasmid, pACYC184, was developed. Using this two-plasmid system, R3HB could be efficiently produced with a negligible amount of PHB left over (Table 1; *[18]*).

Conclusion

As indicated, R3HA could be efficiently produced by employing natural PHA producers or metabolically engineered *E. coli* harboring the genes involved in the biosynthesis and degradation of PHA. Since more than 150 different monomer units (RHAs) have been found to be incorporated into PHAs *(5)*, these RHAs can be produced by employing the same strategy. The biosynthesis described here is a good example of "green chemistry" since high-value fine chemicals and enantiomerically pure hydrocarboxylic acids are produced from renewable resources such as glucose and fatty acids.

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