Microbial synthesis and enzymatic degradation of renewable poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate]

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Abstract
This paper reports our recent progress on a microbial system for efficient production of biodegradable copolyester of poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate], P(3HB-co-3HHx), with desirable copolymer compositions using genetically engineered bacteria. We have developed a fermentation technique to achieve high cell density cultivation of a recombinant \textit{Ralstonia eutropha} using inexpensive soybean oil as a sole carbon source. In addition, we demonstrate that the use of polyhydroxyalkanoate (PHA) synthase (PhaC) mutants having an amino acid substitutions is able to vary the copolymer composition of P(3HB-co-3HHx) synthesized from soybean oil by the recombinant bacteria. On the other hand, it is also important to understand the enzymatic degradation process of PHA materials for establishing a method to regulate the rate of biodegradation. The enzymatic degradation process of P(3HB-co-3HHx) thin film using an extracellular PHB depolymerase has been studied by in situ atomic force microscopy in buffer solution. We demonstrate that the PHA depolymerase predominantly degrades the less-ordered molecular chain-packing regions along the crystallographic $a$-axis.

Keywords: Biodegradable polyester; Microbial synthesis; Polyester crystal; Enzymatic degradation; Atomic force microscopy

1. Introduction

Science and technology of bio-based polymers have experienced a tremendous rise in significance in the last several years. Today, research and development on bio-based polymers are rapidly expanding in both the macromolecular and biological sciences. Bio-based polymers include various polymers produced from renewable resources and CO$_2$, their derivatives, and their blends and composites. Fossil resources are limited, while renewable resources are sustainable.

Polyhydroxyalkanoate (PHA) is a typical bio-based polymer produced from renewable resources such as glucose and vegetable oils by a wide variety of microorganisms as an intracellular storage material of carbon and energy [1,2]. The microbial PHA has attracted much attention as environmentally degradable thermoplastic to be used for a wide range of agricultural, marine, and medical application. A homopolymer containing a repeating unit of (R)-3-hydroxybutyrate, P(3HB) or PHB, is the most common microbial PHA produced in nature. Although, P(3HB) is highly crystalline and has poor elastic quality, (R)-3HB-based copolymer containing a longer chain-length monomer unit (R)-3-hydroxyhexanoate, P(3HB-co-3HHx), exhibits appropriate material properties in flexibility for commercial uses [3]. The material properties of P(3HB-co-3HHx) can be regulated by varying the copolymer composition [1,3].

This paper reports our recent development of a microbial system for efficient production of P(3HB-co-3HHx) with desirable copolymer compositions using recombinant bacteria. A remarkable characteristic of P(3HB-co-3HHx) is its biodegradability. An understanding of the enzymatic degradation process is important for establishing a method to control its biodegradability. The enzymatic degradation process of P(3HB-co-3HHx) thin film is investigated by in situ atomic force microscopy (AFM) in buffer solution.
2. Experimental procedures

2.1. Bacterial strain, plasmids and culture conditions

PHA negative strain of *Ralstonia eutropha* PHB<sup>−</sup>4 (DSM541) was used as host strain for PHA production by the introduction of *Aeromonas caviae* PHA synthase gene (*phaC<sub>A<sub>Ac</sub></sub>*). PHA production was carried out in a mineral salts medium with 2 vol% soybean oil at 30°C [4]. Kanamycin was added to the medium for maintenance of the broad-host-range plasmid in the cells. As for fed-batch culture using a 10 l-jar fermentor, the initial working volume was set at 5 l [5]. The pH and dissolved oxygen (DO) concentration in the culture liquid were controlled using a pH electrode and a DO electrode, respectively. After cultivation, collected cells were washed with water/hexane to remove the remaining soybean oil, and then lyophilized. The PHA content in dry cells and the composition were determined by gas chromatography after methanolysis of lyophilized cells in the presence of 15% sulfuric acid.

Recombinant plasmids for functional expression of wild-type *phaC<sub>A</sub>* gene or its mutants in *R. eutropha* PHB<sup>−</sup>4, pBBREE32d13 and pJRDE32d13, were constructed using the broad-host-range plasmids pBBR1MCS-2 and pRDE215, respectively [4,6]. These plasmids contain *phaC<sub>A<sub>Ac</sub></sub>* gene with its native promoter region.

2.2. AFM observation

Cast thin film of biosynthesized P(3HB-co-5 mol% 3HHx) of 100 nm thickness was prepared on glass substrates by solvent-cast technique, as reported previously [7–12]. The cast thin film was melted at 180°C, and then crystallized at 110°C for 1 day. The surface morphologies of P(3HB-co-5 mol% 3HHx) thin film were observed by AFM (SEIKO Instruments Inc., SPI3800/SPA400) in air at 25°C. Si tip mounted on 200 μm long cantilever with spring constants of 1.5 N/m was operated in the dynamic force (tapping) mode in phosphate buffer solution. For the AFM observation in buffer solution, the interaction force between the sample surface and cantilever tip was reduced in order to avoid the damage on the lamellar surface by cantilever tip [12]. Simultaneous registration was performed for height and deflection images.

3. Results and discussion

3.1. Efficient production of P(3HB-co-3HHx) using recombinant bacteria

Table 1 lists the cultivation results of recombinant *R. eutropha* harboring a wild-type *phaC<sub>A</sub>* from *A. caviae*.

| Plasmid introduced (reactor) | Culture time (h) | Dry cell weight (g/l) | PHA content (wt%) | PHA composition | Molecular weight | Productivity (g-PHA/g-soybean oil) | Yield (g-PHA/g-soybean oil) |
|-----------------------------|-----------------|----------------------|------------------|----------------|-----------------|-------------------------------|--------------------------|
| (500 ml-shaking-flask)      |                 |                      |                  |                |                 |                               |                          |
| pBBREE32d13                 | 72              | 3.2                  | 83               | 96             | 4               | 1.8                           | 0.037                    | –                        |
| pJRDE32d13                  | 72              | 2.2                  | 78               | 96             | 4               | –                             | 0.024                    | –                        |
| (10 l-jar fermentor)        |                 |                      |                  |                |                 |                               |                          |
| pJRDE32d13                  | 96              | 138                  | 74               | 95             | 5               | 2.5                           | 1.06                     | 0.72                     |

Vegetable oils are desirable feedstock for PHA production by bacteria because of their low material costs (30 US cents per kg) and relatively high yields of PHA production (0.7–0.8 g-PHA per g-vegetable oil; this value is two-fold higher than that from glucose) [2,13]. The bacterium *A. caviae* isolated from soil is capable of producing P(3HB-co-3HHx) copolymer from vegetable oils [3]. However, this bacterium has a poor capability for PHA accumulation (less than 30 wt% of the dry cells). The well-known PHA producing bacterium *R. eutropha* is capable of accumulating PHA at high levels exceeding 80 wt% of the dry cells, but it produces only P(3HB) homopolymer from vegetable oils. Hence, a genetically engineered recombinant *R. eutropha* PHB<sup>−</sup>4 (PHA negative mutant strain) harboring *A. caviae* PHA synthase gene (*phaC<sub>A<sub>Ac</sub></sub>* has been generated [4].

Table 1 P(3HB-co-3HHx) production from soybean oil at 30°C by recombinant *R. eutropha* harboring a wild-type *phaC<sub>A<sub>Ac</sub></sub>* from *A. caviae*.
Thus, these recombinants exhibited favorable characteristics of P(3HB-co-3HHx) synthesis with a high PHA content from soybean oil.

In order to attain a high cell concentration in culture, larger scale fermentation using a 10 l-jar fermentor was carried out, and the fermentation conditions were controlled by a computer system [5]. The soybean oil concentration in the fermentor was kept at 20 g/l because this concentration was the most preferable level for cell growth. In addition, a phosphorous-limitation technique in culture liquid was used to initiate an efficient PHA accumulation in cells after exponential growth phase. By keeping soybean oil concentration at 20 g/l and employing phosphorous-limitation strategy, we succeeded in achieving efficient production of P(3HB-co-5 mol% 3HHx) with high cell concentration of 138 g/l and high PHA content of 74 wt%. The PHA yield was as high as 0.72 g-PHA per g-soybean oil (Table 1).

In a previous paper [13], we reported an environmental life cycle comparison of PHA production from glucose and soybean oil by bacterial fermentation on the basis of life cycle inventory analysis on total fossil energy consumptions and net CO2 emissions. It has been concluded that the net CO2 emissions of PHA production from soybean oil (less than 1.0 kg-CO2/kg-PHA) are relatively lower than those for production of petrochemical plastics such as polyethylene, polypropylene, polystyrene and polyethylene terephthalate (1.7–3.1 kg-CO2/kg-plastic). In addition, high yield production of PHA from soybean oil was verified to be cost competitiveness (less than 4 US$/kg-PHA).

3.2. Compositional regulation in P(3HB-co-3HHx) production by evolutionary engineering

It may be possible to regulate copolymer composition in P(3HB-co-3HHx) by microbial production feeding co-substrates such as butyric acid and hexanoic acid together with vegetable oils. However, bacterial growth is limited by the presence of these organic acids, resulting in the low polymer productivity. In addition, the costs of organic acids are relatively expensive. Therefore, a new method to regulate copolymer composition has been needed.

In order to regulate copolymer composition, we attempted to employ mutated PHA synthases of PhaCAc. In a previous study [14], we generated two higher active mutants of PhaCAc, E2-50 and T3-11 (56 and 21% increases in activity compared with the wild-type enzyme, respectively), by evolutionary engineering. During the course of this evolutionary engineering research, a lot of lower active mutants were also generated. By introducing the genes of these higher and lower active mutants into R. eutropha PHB-4, P(3HB-co-3HHx) production was carried out using soybean oil as a sole carbon source. Fig. 1 shows the 3HHx fraction in P(3HB-co-3HHx) produced by the recombinant strains. Interestingly, the 3HHx fraction in P(3HB-co-3HHx) synthesized by the higher active mutants was increased up to 5.1 mol% compared with that (3.5 mol%) by the wild-type, while the lower active mutants produced copolymers with low 3HHx fractions. Thus, this result demonstrates the feasibility of regulating the copolymer composition in P(3HB-co-3HHx) production by use of appropriate mutated PhaCAc.

3.3. Enzymatic degradation of P(3HB-co-3HHx) thin film

PHA materials are hydrolyzed by extracellular PHB depolymerases which are secreted from various kinds of microorganisms [15]. In our group, the enzymatic degradation behavior of PHA lamellar crystals by some PHB depolymerases has been studied on the surface of melt-crystallized PHA thin films [8,10–12]. AFM is a powerful tool to observe the morphological change of
lamellar crystals during the enzymatic degradation process in real time under an aqueous environment.

The P(3HB-co-5 mol% 3HHx) thin film of about 100 nm thickness was crystallized at 110 °C for 1 day after melting at 180 °C and kept at room temperature. As reported in previous paper [10], lamellar crystals of 8-10 nm thickness are formed during the primary crystallization process at 110 °C, and thin surface layers of 4-6 nm thickness are often generated during storage at room temperature around the lamellar crystals of 8-10 nm thickness.

Fig. 2 shows the time-dependent AFM images of P(3HB-co-5 mol% 3HHx) thin film before and during enzymatic degradation by PHB depolymerase from *R. pickettii* T1. As shown in Fig. 2A, before enzymatic degradation, two types of crystals are observed on the film surface consisting of lamellar crystals of 8–10 nm thickness and thin surface crystals of 4–5 nm thickness. After addition of the enzyme into buffer solution, morphological changes of film surface during enzymatic degradation were directly observed in the phosphate buffer solution with enzyme (the final concentration was ca. 1.0 μg/ml). At the initial stage of enzymatic degradation, surface thin layers of 4–5 nm thickness formed at room temperature were completely eroded by the enzyme (Fig. 2B), while thicker lamellar crystals of 8–10 nm thickness remained almost unchanged, suggesting that thinner crystals are preferentially hydrolyzed by PHB depolymerase.

The lamellar crystals of 8–10 nm thickness were subsequently eroded by the function of PHB depolymerase as shown in Fig. 2C–F. The lamellar crystals were hydrolyzed along the crystallographic *a*-axis, while the lamellar thickness remained almost unchanged through the enzymatic degradation process. These results indicate that enzymatic degradation takes place predominantly from the edge and end of the crystal rather than the chain-folding surface. As a result of crystal erosion along the *a*-axis, splintered morphologies were generated at the tips of lamellar crystals as shown at arrows in the AFM images. The widths of fibriller crystals generated during the enzymatic degradation were ranged from 30 to 80 nm.

Similar enzymatic degradation manner along the crystallographic *a*-axis has been found on the surface of thin films of other PHAs: poly[(R)-3HB-co-(R)-3-hydroxyvalerate] and poly[(R)-3HB-co-6-hydroxyhexanoate] [11,12]. In some cases, lamellar crystals in the PHA thin films were also eroded at the middle part of the crystal along the crystallographic *b*-axis, resulting in the formation of small crevices. From the crevices, enzymatic degradation proceeded along the *a*-axis. These results suggest that lamellar crystals are composed of tight chain-packing regions and less ordered chain-packing regions, and that the defective regions along both the longitudinal (*a*-axis) and lateral (*b*-axis) directions of the crystal are preferentially eroded by PHB depolymerase. Accordingly, it is suggested that the enzymatic degradation process is consisted of two schemes; one is the degradation along the *a*-axis, resulting in the formation of splintered crystal morphology with 30–80 nm intervals, and the other is hydrolysis along the *b*-axis creating some crevices, from which the following

![Fig. 2. Continuous AFM deflection images of P(3HB-co-5 mol% 3HHx) thin film before (A) and during enzymatic degradation by PHB depolymerase from *R. pickettii* T1 (B–F). The first frame (A) was obtained in phosphate buffer solution at 20 °C, while the following frames (B–F) were taken during enzymatic degradation for 17 min (B), 49 min (C), 1 h 20 min (D), 1 h 49 min (E), and 2 h 15 min (F), respectively. Arrows in the images indicate the typical lamellar crystals with splintered morphology along the crystallographic *a*-axis. In the frame (A), the arrow (a) shows the typical lamellar crystal of 8–10 nm thickness, while the arrow (b) indicates the thin surface crystal of 4–5 nm thickness.](image-url)
degradation proceeds along the \( \alpha \)-axis. The quantitative studies on the enzymatic degradation behavior of PHA lamellar crystals are in progress by using the real-time AFM technique.

4. Conclusions

Development of bio-based polymers would be one of key-technologies to build a sustainable society. In order to achieve this scientific object, we have developed a microbial system for effective production of \( \text{P}(3\text{HB-co-3HHx}) \) from inexpensive vegetable oil with desirable copolymer composition by means of culture-, genetic- and evolutionary-engineerings. Furthermore, this production system has been demonstrated to be an environmentally friendly one by life cycle inventory analysis. On the other hand, an understanding of the enzymatic degradation process on PHA materials is important for controlling its biodegradability. The enzymatic degradation process of \( \text{P}(3\text{HB-co-3HHx}) \) thin film using an extracellular PHB depolymerase has been studied by in situ AFM technique in solution.

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