Engineering bacteria for enhanced polyhydroxyalkanoates (PHA) biosynthesis

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A R T I C L E   I N F O

Article history:
Received 20 August 2017
Received in revised form 16 September 2017
Accepted 16 September 2017

Keywords:
PHB
Polyhydroxyalkanoates
Extremophiles
Halophiles
Next generation industrial biotechnology
NGIB
Metabolic engineering
Pathway engineering
Morphology engineering

A B S T R A C T

Polyhydroxyalkanoates (PHA) have been produced by some bacteria as bioplastics for many years. Yet their commercialization is still on the way. A few issues are related to the difficulty of PHA commercialization: namely, high cost and instabilities on molecular weights (Mw) and structures, thus instability on thermo-mechanical properties. The high cost is the result of complicated bioprocessing associated with sterilization, low conversion of carbon substrates to PHA products, and slow growth of microorganisms as well as difficulty of downstream separation. Future engineering on PHA producing microorganisms should be focused on contamination resistant bacteria especially extremophiles, developments of engineering approaches for the extremophiles, increase on carbon substrates to PHA conversion and controlling Mw of PHA. The concept proof studies could still be conducted on E. coli or Pseudomonas spp. that are easily used for molecular manipulations. In this review, we will use E. coli and halophiles as examples to show how to engineer bacteria for enhanced PHA biosynthesis and for increasing PHA competitiveness.

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1. Introduction

Polyhydroxyalkanoates (PHA) have been produced since the 1980s with limited market success [1–3]. Many challenges are related to the limited PHA commercialization (Table 1), especially the high production cost and instability on thermo-mechanical...
properties resulted from unstable molecular weights (Mw) and structures, that are also associated with unstable PHA synthase activity and monomer supplies [4–10]. Efforts have been made to meet these challenges [5,11–13].

The high cost is the result of high energy demand related to complicated sterilization and intensive aeration, low conversion of carbon substrates (S) to PHA products (P), slow growth of microorganisms, discontinuous processes and expensive downstream processing et al. (Table 1) [14,15]. The use of extremophilic bacteria combined with metabolic engineering and synthetic biology could fully address these issues [16,17].

Future engineering on PHA producing microorganisms should be focused on contamination resistant bacteria especially extremophiles, developments of engineering approaches for the extremophiles (which is called “Next Generation Industry Biotechnology” or “NGIB”, which will be discussed in section 6 in this paper), increase on carbon substrates to PHA conversion and controlling Mw of PHA (Table 1). The concept proof studies could still be conducted on E. coli or Pseudomonas spp. that are easily used for molecular manipulations. In this review, we will use E. coli, Pseudomonas spp., and halophiles as examples to show how to engineer bacteria for better PHA biosynthesis and for increased PHA application competitiveness.

2. Redirecting substrates to PHA conversion pathways

In many cases, substrates are the most important factor for high production cost. This is especially true for PHA production [11]. For example, the production of PHA containing non 3-hydroxybutyrate (3HB) monomers requires fatty acid(s) as substrate for formation of other non 3HB short-chain-length (scl) or medium-chain-length (mcl) monomers [25–29]. Since most fatty acids will be beta-oxidized to acetyl-CoA for the uses of many other biosynthesis pathways other than for PHA synthesis, it wastes a lot of expensive fatty acids for generating acetyl-CoA. It can be a problem when low-cost glucose is used. Due to the beta-oxidation, fatty acid substrates conversion to PHA products are very low, resulting in high cost of PHA production.

The substrate to PHA conversion efficiency has been significantly improved with the deletions of enzymes FadA and FadB in the beta-oxidation pathway of Pseudomonas putida (Fig. 1), as fatty acid substrates were mostly converted into 3-hydroxyacyl-CoA for PHA synthesis instead of being oxidized to become acetyl-CoA. Beta-oxidation pathway deleted Pseudomonas spp. have been reported to produce PHA containing 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydecanedioate (3HDD) in the forms of homopolymers, block- or random copolymers [32].

A metabolically engineered Escherichia coli has been constructed by co-expressing genes involved in succinate degradation in Clostridium kluyveri and P(3HB) accumulation pathway of Raistonia eutropha. This engineered E. coli can produce poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] from glucose. Also, E. coli native succinate semialdehyde dehydrogenase genes sad and gabcd were both deleted to enhance the carbon flux to 4HB biosynthesis [18]. Povolo et al. [34] reported that the production of P(3HB-co-3HV-co-4HB) terpolymer can be obtained directly by the use of lactose or waste raw materials such as cheese whey as carbon sources. Cerrone et al. [35] demonstrated the use of a mannitol rich enelled grass press juice (EGP) as a renewable carbon substrate for poly(hydroxyalkanoates) (PHA) production. Fed-batch cultivations of Burkholderia sacchari IPT101 using EGP as sole carbon source produced 44.5 g/L CDW containing 33% poly-3-hydroxybutyrate (PHB) in 36 h. Park et al. [36] constructed a su-crolose utilization pathway in Ralstonia eutropha NCIMB11599 and R. eutropha 437–540 by introducing the Mannheimia succiniproduc- dences MBEL552 sacC gene that encodes β-fructofuranosidase. β-fructofuranosidase extracted into the culture medium could hydrolyze sucrose to glucose and fructose, which efficiently used sucrose as carbon sources by recombinant R. eutropha strains. A high P(3HB) content of 73.2 wt% was obtained when R. eutropha NCIMB11599 was cultured in nitrogen-free chemically defined medium containing 20 g/L of sucrose [37].

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3. Stabilization of PHA monomer ratios and molecular weights (Mw)

Due to the fluctuation of PHA synthase activity and monomer supplies, monomer fluctuations in copolymers and PHA Mw vary from batch to batch, this is not desirable by any consumer. Therefore, a lot of efforts have been made to stabilize the PHA monomer structures and Mw. Tripathi et al. [19] used the above beta-oxidation deleted Pseudomonas putida KT2442 as a platform for the biosynthesis of polyhydroxyalkanoates with adjustable monomer contents and compositions. The monomer ratios can be precisely controlled by feeding fatty acids with a predefined ratio (Fig. 2). They achieved to prepare random copolymers PHBHHx or block copolymers consisting of precisely adjustable 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx). The materials thus showed stable properties if the monomer ratios were stable [19,38].

Similarly, Wang Ying et al. [39] succeeded in synthesizing homopolymers of C5 (3-hydroxyvalerate) to C14 (4-hydroxytetradecanoate) using beta-oxidation deleted P. entomophila LAC23 grown on different fatty acids as precursors, respectively. The examples clearly demonstrate that beta-oxidation deleted mutants can help control PHA monomer structures, as also evidenced by several other studies [40].
Some factors have a direct impact on the molecular weight of PHA. Such as the concentration (or activity) of PHA synthase, the occurrence of a chain transfer reaction, the catalytic activity of PHA synthase and the simultaneous degradation of PHA during biosynthesis [41]. PHA molecular weight needs to be controlled to tailor the physical properties of the polymer. PHA molecular weight can be reduced via the addition of chain transfer agents such as poly(ethylene glycol) (PEG), methanol, ethanol and isopropanol to the culture medium during production and mutations in the N-terminus of PHA synthase [42,43]. Moreover, PHA molecular weights can be controlled by adjusting PHA synthase activity. CRISPRi (Clustered regularly interspaced short palindromic repeats interference) was able to control the \textit{phaC} transcription and thus PhaC activity. Li Dan et al. [10] found that PHB content, molecular weight and polydispersity were approximately in direct and reverse proportion to the PhaC activity, respectively. Very importantly, a higher PhaC activity led to more intracellular PHB accumulation yet with less PHB molecular weights and wider polydispersity. PHB contents could be controlled in the ranges of 2.0—75% cell dry weights, molecular weights from 2 to 6 millions Dalton and a polydispersity of 1.2—1.43 in 48 h shake flask studies. This Mw control studies should be further confirmed in large scale fermentor studies.

4. Acceleration of cell growth

Most bacteria are grown in a common binary fission way (Fig. 3). Wu Hong et al. [21] changed the binary fission to multiple fission by deleting fission related genes \textit{minC} and \textit{minD} together, leading to the formation of multiple fission rings (Z-rings) in several positions of an elongated cell, achieving cell division into more than two daughter cells at same time (Fig. 3). In addition, some genes related to cell division process including \textit{ftsQ}, \textit{ftsL}, \textit{ftsW}, \textit{ftsN} and \textit{ftsZ}, together with the cell shape control gene \textit{mreB}, were all overexpressed in \textit{E. coli} JM109 \textit{ΔminCD} to further improve cell growth and PHA production. This resulted in more cell dry weights (CDW) and more than 80% polyhydroxybutyrate (PHB) accumulation increases compared to its binary fission control. This study demonstrates that changing the cell division pattern and cell morphology help accelerate cell growth and PHB accumulation. In another related study, Wu Hong et al. [44] further demonstrated that a combination of the multiple division pattern with elongated cell shape of \textit{E. coli} improved PHB production. In addition, Tyo group [45] developed a toggle switch that uses glucose sensing to decouple growth and production phase strategy. This industrially relevant auto-inducible genetic switch responds to glucose availability to precisely time the expression of burdensome pathway enzymes for enhanced bio-production, which improved growth by 2-fold with comparable PHB production yields to a constitutively expressing system. Those provided a new vision for enhanced PHA production.

5. Bacterial morphology engineering for easy downstream separation

Tiny bacterial cell sizes create complexity for downstream
separation. Morphology engineering changes cell sizes and shapes, allowing easy downstream separation [24]. Genes *ftsZ* and *mreB* encoding proteins of bacterial fission ring and skeletons, respectively, are essential for cell growth and for maintaining the bacterial shapes [46–50]. Clustered regularly interspaced short palindromic repeats interference (CRISPRi), was used to regulate expression intensities of *ftsZ* and/or *mreB* in *E. coli* resulting in various reduced expression levels of *ftsZ* or/and *mreB*, respectively [51]. It was found that the stronger the repression on genes *ftsZ* or/and *mreB*, the longer the *E. coli* fibers, and the larger the *E. coli* cells [51]. Combined repressions on expressions of *ftsZ* and *mreB* generated long and larger *E. coli* with diverse morphologies including various sizes of gourds, bars, coccus, spindles, multi-angles and ellipsoids (Fig. 4). In all cases, PHB accumulations were improved. Enlarged morphology increased PHB synthesis from 40% to 80% PHB, it also promotes gravity separation of cells from fermentation broth [23,24,52].

**Fig. 3.** The common bacterial binary fission can be changed to multiple fission by deleting fission related genes *minC* and *minD* together, leading to the formation of multiple fission rings (Z-rings) in several positions of an elongated cell, achieving cell division into more than two daughter cells.

**Fig. 4.** CRISPRi was used to regulate expression intensities of *ftsZ* or/and *mreB* in *E. coli* resulting in various reduced expression levels of *ftsZ* or/and *mreB*, respectively [51]. Combined repressions on expressions of *ftsZ* and *mreB* generated long and larger *E. coli* with diverse morphologies and enhanced PHB accumulations. Large cells are prone to separate from broth via gravity or filtration.
6. Future prospects: next generation industry biotechnology (NGIB) based on extremophiles (halophiles)

Extremophiles grown under extreme conditions are more resistant to microbial contamination. Among them, halophilic bacteria are able to grow rapidly in medium with high salt concentrations under high pH [16], they are thus contamination resistant as few other microorganisms can do so. Halophilic bacteria were found able to grow contamination free in open unsterile and continuous processes in seawater medium for at least two months [53]. Their values could be further improved by introducing new pathways or adding new genetic parts.

However, genetic parts are often influenced by host strains, and altered activity of biological parts frequently causes failures in process control [54,55]. Therefore, in order to fully realizing the potential of *Halomonas*, genetic parts with a tight regulation and high efficiency need to be developed. Recently, Technology has also been developed for genetic manipulation of halophilic bacteria [22,56]. Molecular engineering tools have been developed to construct recombinant *Halomonas* spp. for production of foreign protein [57], small molecular compound 5-aminolevulinic acid [58], PHB homopolymers consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) with high substrate to PHA conversion efficiency [22,59,60]. More engineering studies will lead to the generation of new products produced by engineered halophiles.

The biotechnology based on extremophiles grown under open unsterile conditions will surely promote the emerging “Next Generation Industrial Biotechnology” or NGIB for bio-production with reduced cost and thus improved competitiveness.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This research was financially supported by a grant from Ministry of Sciences and Technology (Grant No. 2016YFB0302504), and grants from National Natural Science Foundation of China (Grant No. 31430003), Tsinghua President Fund also supported this project (Grant No. 2015THZ10).

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