

Jinyu Zhang · Ning Hao · Guo-Qiang Chen

Effect of expressing polyhydroxybutyrate synthesis genes (*phbCAB*) in *Streptococcus zooepidemicus* on production of lactic acid and hyaluronic acid

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Abstract Hyaluronic acid (HA) has been industrially produced using the gram-positive bacterium *Streptococcus zooepidemicus*. Large amount of lactic acid formation was one of the important factors that restricted cell growth and HA productivity and lowered the substrate to HA conversion efficiency in a fermentor. In this study, polyhydroxybutyrate (PHB) synthesis genes (*phbCAB*) of *Ralstonia eutropha* were cloned from the plasmid pBHR68 and were inserted into the plasmid pEU308, an expression vector for gram-positive bacteria. The plasmid was transformed into *S. zooepidemicus* by electroporation. β -Ketothiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and polyhydroxyalkanoate (PHA) synthase (PhbC) activity assays were carried out to demonstrate the expression of these genes. The PhbA and PhbB activities were 3.13 and 1.23 U mg⁻¹, respectively. No PhbC activities were detected. In shake flask studies, there was no obvious difference between the wild-type and recombinant *S. zooepidemicus* harboring *phbCAB* genes in terms of lactic acid and HA formation. However, in fermentor studies, the recombinant produced only 40 g L⁻¹ lactic acid and 7.5 g L⁻¹ HA, whereas the wild type produced 65 g L⁻¹ lactic acid and 5.5 g L⁻¹ HA. These results suggested that expression of *phbCAB* genes in *S. zooepidemicus* could help regulate HA production metabolism. Because the lactic acid formation in *S. zooepidemicus* was sensitive to cellular oxidation/reduction potential, it is proposed that the PHB synthesis pathway could act as a regulator to adjust the cellular oxidation/reduction potential. This is the first study demonstrating that PHA synthesis related to energy and carbon metabolism could be employed as a pathway to regulate other cellular metabolism and possibly to regulate the production of other metabolic products.

Introduction

Streptococcus equi subsp. *zooepidemicus* is a Lancefield group C *Streptococcus* and a common animal pathogen. It infects domestic and laboratory animals and has been found to cause respiratory infections in horses and, infrequently, mastitis in cows (Kester et al. 1993). In addition, its nonpathogenic mutant is widely used in industrial hyaluronic acid (HA) production (Nimrod 1988).

HA is a high molecular mass glycosaminoglycan composed of D-glucuronic acid and N-acetyl-D-glucosamine. It is present in various connective tissues of animals, such as skin and cartilage (O'Regan et al. 1994). In addition, HA is produced by various microorganisms such as streptococci types A and C. About 10% of the consumed carbon is used for the biosynthesis of HA (Holmstrom and Ricica 1967). Construction of one repeat unit needs two NAD factors, five ATP equivalents, and one acetyl-CoA group.

In fermentation processes, *S. zooepidemicus* produces a large amount of lactic acid, which consumes a great deal of carbon source and is harmful to the cells; yet, this is necessary for the bacterium to reduce the toxicity of pyruvate accumulation (Jeffrey and Jacky 1996). Two NADHs are oxidized to NAD when two pyruvates are converted to lactic acid by lactic acid dehydrogenase. Deletion of this enzyme is lethal to the bacterium (Chen et al. 1994). Overexpression of the NADH oxidase gene can reduce the lactic acid formation (Chong and Nielsen 2003a,b). In fermentation studies, it was found that lactic acid concentration was reduced when the dissolved oxygen (DO) was maintained at a higher level compared with that at a lower level (Gao 1999). This suggests that the lactic acid pathway plays an important role in generating cellular oxidation potential.

The synthesis of polyhydroxybutyrate (PHB) in *Ralstonia eutropha* has been investigated for many years. The operon for the PHB synthesis is composed of three genes, *phbA*, *phbB*, and *phbC*, which respectively encode the enzymes β -ketothiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and PHB synthase (PhbC). NADPH is oxidized to NADP during PHB synthesis (Lee et al. 1999).

J. Zhang · N. Hao · G.-Q. Chen (✉)
Department of Biological Sciences and Biotechnology,
Tsinghua University,
Beijing, 100084, China
e-mail: chengq@mail.tsinghua.edu.cn
Tel.: +86-10-62783844
Fax: +86-10-62788784

This pathway could be used to provide oxidation/reduction potential for the cells, and it could possibly affect microbial metabolic products.

For the first time, we used the PHB synthesis operon to regulate lactic acid and HA production by *S. zooepidemicus*.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

S. zooepidemicus was cultivated in THYB [Todd–Hewitt (TH) broth (OXOID) supplemented with 0.5% yeast extract] at 37°C. *Escherichia coli* cells were grown in Luria–Bertani (LB) medium at 37°C. The concentration of spectinomycin was 100 µg mL⁻¹ for *S. zooepidemicus* and *E. coli*. The concentration of isopropyl-L-thio-β-D-galactopyranoside (IPTG) was 20 mM. Plasmid pBHR68 (Spiekermann et al. 1999), containing the entire *phbCAB* operon of *R. eutropha*, and gram-positive bacterial expression vector pEU308 (Eichenbaum et al. 1998) were used in this study.

The fermentation experiments were conducted in a chemically defined medium (Gao 1999). The carbon sources for flask fermentation and fermentor studies were 40 and 70 g L⁻¹ sucrose, respectively. Cells from the THYB plate were inoculated into a seed medium. After incubation at 37°C, they were used to inoculate the fermentor (Bioflo 3000, New Brunswick Scientific Co. Inc., NJ, USA) during the early exponential growth phase.

A 7.5-L Bioflo 3000 fermentor (New Brunswick Scientific) with an operating volume of 3 L was used. The pH in the culture was controlled at 7.0 by automatic addition of 5 mol L⁻¹ sodium hydroxide solution. The agitation rate was 200 rpm at the beginning. When the DO was decreased to 0, the agitation rate increased to 500 rpm. When the DO was again reduced to 0, the agitation rate

increased to 650 rpm and was so maintained up to the end of the fermentation. The fermentor was sparged with a continuous supply of air (5 L min⁻¹) during cultivation.

Plasmid construction

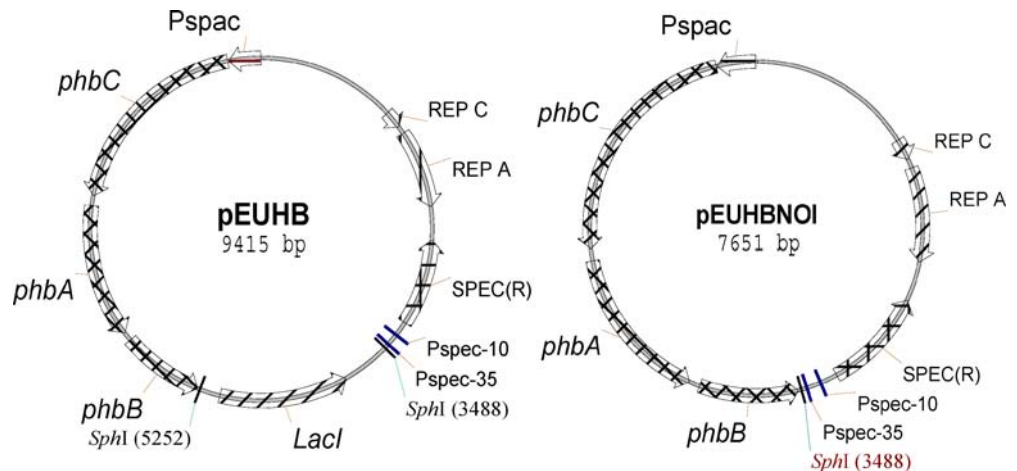
The plasmid construction is shown in Fig. 1. *phbCAB* genes were amplified from pBHR68 by PCR using the following primers: P_{*phbCAB*-up}, ATAAAGCTT (*Hind*III) AAGGAGGATGGCGACCGGCAAAGGC; P_{*phbCAB*-down}, GTTATCGAT (*Cl*aI) CGGCAGGTCAGCCCATAT. The conditions for PCR amplification using *Taq* DNA polymerase with the primers were as follows: 96°C for 4 min, 30 cycles of 96°C for 45 s, 66°C for 1 min, and 72°C for 4.5 min, followed by 72°C for 10 min on a thermal cycler (Mastercycler gradient, Eppendorf, Germany). Plasmid pEUHB was generated by ligating the *Hind*III–*Cl*aI fragment of the PCR product to plasmid pEU308 cut with *Hind*III and *Cl*aI, creating a transcriptional fusion of the *Spac* promoter to the *phbCAB* gene. Plasmid pEUHBNOI was generated by self-ligation of pEUHB after digestion with *Sph*I, which removed the *lacI*^r repressor gene (Yansura and Henner 1984).

Competent *E. coli* JM109 cells were prepared according to the method described by Sambrook and Russell (2001). Competent cells were transformed with ligation mixtures and selected on LB agar plates containing spectinomycin (100 µg mL⁻¹). The newly constructed plasmid was identified by restriction endonuclease digestion and agarose gel electrophoresis.

Electroporation of *S. zooepidemicus*

S. zooepidemicus cells were grown in THYB. After 14 h of incubation, cells were inoculated into 50 mL fresh THYB

Fig. 1 Schematic representation of the plasmid pEUHB and pEUHBNOI. *phbCAB* genes were cloned under the control of the *Spac* promoter in plasmid pEUHB. The *lacI*^r repressor gene was rejected in the plasmid pEUHBNOI



to less than 0.05 at OD 660 nm. The cells were further grown to about 0.25 at OD 660 nm. Approximately 30 min prior to harvest, 20 mg of hyaluronidase was added, followed by collection of the pellet cells at $8,000\times g$ and 4°C for 10 min. The supernatant was discarded, and the pellets were washed in 20 mL of 0.5 M sucrose solution. After centrifugation, the cells were washed again in 1 mL of 0.5 M sucrose solution. Finally, the cells were resuspended in 250 μL of 10% glycerol and 0.5 M sucrose solution, followed by dividing into aliquots of useful lots. Competent cells were stored at -80°C .

About 500–5,000 ng plasmids was added into 200 μL of competent cells. The mixture was removed immediately and placed into an ice-cold cuvette. The DNA should be in a very low salt buffer. The voltage for the transformation was 1.67 kV mm^{-1} . After application of the electric pulse, the mixture was immediately placed into 10 mL of fresh THYB and incubated on ice for 30–60 min. Then, it was incubated in 37°C water bath for 1 h. After incubation, the cells were concentrated by centrifugation at $6,000\times g$ and 14°C for 10 min, followed by resuspension in 1 mL of THYB and plating on selective media. Colonies (colony-forming units) should be present in 24 h.

Assay of enzyme activities of β -ketothiolase, acetoacetyl-CoA reductase, and polyhydroxyalkanoates synthase

For analysis of the enzyme activity of PhbA and PhbB, cells were harvested by centrifugation after 12 h of cultivation, washed once with 0.1 mol L^{-1} Tris-HCl (pH 7.5), and resuspended in a 10% volume of the same buffer. Crude extract was prepared from the supernatant after cells were disrupted by sonication and centrifugation. Protein content in the crude extract was determined by the method of Bradford using Bio-Rad (Hercules, CA, USA) protein assay solution and bovine serum albumin as the standard (Bradford 1976).

The activity of PhbA was determined by a photometer assay at 25°C , calculating the decrease of absorption of acetoacetyl-CoA at 303 nm, as described by Nishimura et al. (1978). PhbB reductase activity was determined by a photometer assay at 25°C based on the decrease of absorption of NADPH at 340 nm (Senior and Dawes 1973), and PhbC activity was determined spectrophotometrically by monitoring the release of CoA at 412 nm, as described by Rehm et al. (2002).

Quantitation of hyaluronic and lactic acid

HA concentration was determined by the Bitter–Muir method (Bitter and Muri 1962). The concentration of lactic acid in the supernatant was determined by ion exchange HPLC (AS3000, SpectraSYSTEM, USA) equipped with a $7.8\times 300\text{ mm}$ HPX-87H ion exclusion column (Bio-Rad). The mobile phase consisted of $0.005\text{ M H}_2\text{SO}_4$ pumped at a flow rate of 0.5 mL min^{-1} . The column was maintained at

40°C . The peak elution profile was monitored with a refractive index detector (RI-150, SpectraSYSTEM).

Results

Expression of *phbCAB* gene in *S. zooepidemicus*

The plasmids harboring *phbCAB* genes were transformed into *S. zooepidemicus* by electroporation. In the preparation of competent cells, the addition of glycine dramatically increased the transformation efficiency; yet, it led to a sharp decline in HA production. Thus, glycine was omitted in the experiments. To verify the PhbA and PhbB enzyme activities, *S. zooepidemicus* harboring the plasmid pEUHB was incubated with 20 mM IPTG for 12 h. The enzyme activities were studied with the procedure described in “Materials and methods.” The results are shown in Table 1. The wild-type strain did not show any PhbA and PhbB activity; in comparison, the activities of these two enzymes in recombinant strain were 3.13 and 1.23 U mg^{-1} , respectively. Interestingly, no PhbC activity was detected either in the wild-type or in the recombinant strain. As a result, no PHB accumulation in the recombinant was detected by gas chromatography.

Influence of *phbCAB* expression on the production of hyaluronic acid and lactic acid in shake flask cultures

S. zooepidemicus was cultured in sucrose (40 g L^{-1}) at 37°C for 14 h. There was almost no difference between the wild-type and the recombinant strains in terms of cell dry weight and production of lactic acid and HA. These data showed that there was no remarkable influence of *phbCAB* expression under flask cultivation conditions (Table 2).

Table 1 Enzyme activity assay of β -ketothiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and polyhydroxyalkanoate (PHA) synthase (PhbC)

| Strain | PhbA activity (U mg^{-1}) ^a | PhbB activity (U mg^{-1}) ^b | PhbC activity (U mg^{-1}) ^c |
|--------------------|--|--|--|
| Wild-type strain | ND | ND | ND |
| Recombinant strain | 3.13 ^d | 1.23 ^d | ND |

ND Not detected

^aPhbA was monitored at 303 nm by the depletion of acetoacetyl-CoA during the reaction. One unit of PhbA is defined as 1 μmol of acetoacetyl-CoA depletion in 1 min

^bPhbB was monitored at 340 nm by the depletion of NADPH during the reaction. One unit of PhbB is defined as 1 μmol of NADPH depletion in 1 min

^cPhbC was monitored at 412 nm by the release of CoA during the reaction. One unit of PhbC is defined as the amount that produces 1 μmol product in 1 min

^dThis was the average value of two parallel samples

Table 2 Shake flask cultivation of *S. zooepidemicus* harboring different plasmids

| Plasmid | Induction | CDW (g L ⁻¹) | HA (g L ⁻¹) | Lactic acid (g L ⁻¹) |
|----------|-----------|-----------------------------|----------------------------|-------------------------------------|
| — | — | 0.78±0.017 | 0.65±0.1 | 6.6±0.83 |
| PEUHB | None | 0.79±0.02 | 0.62±0.03 | 6.45±0.9 |
| PEUHB | IPTG | 0.76±0.032 | 0.63±0.0058 | 6.43±0.7 |
| PEUHBNOI | None | 0.77±0.017 | 0.6±0.035 | 6.62±0.33 |

Strains were grown on chemical defined medium plus sucrose (40 g L⁻¹) at 37°C for 14 h. IPTG (20 mM) was added at the beginning of inoculation. Spectinomycin (0.1 g L⁻¹) was added to the medium at the beginning

CDW Cell dry weight, HA hyaluronic acid, IPTG isopropyl-L-thio-B-D-galactopyranoside

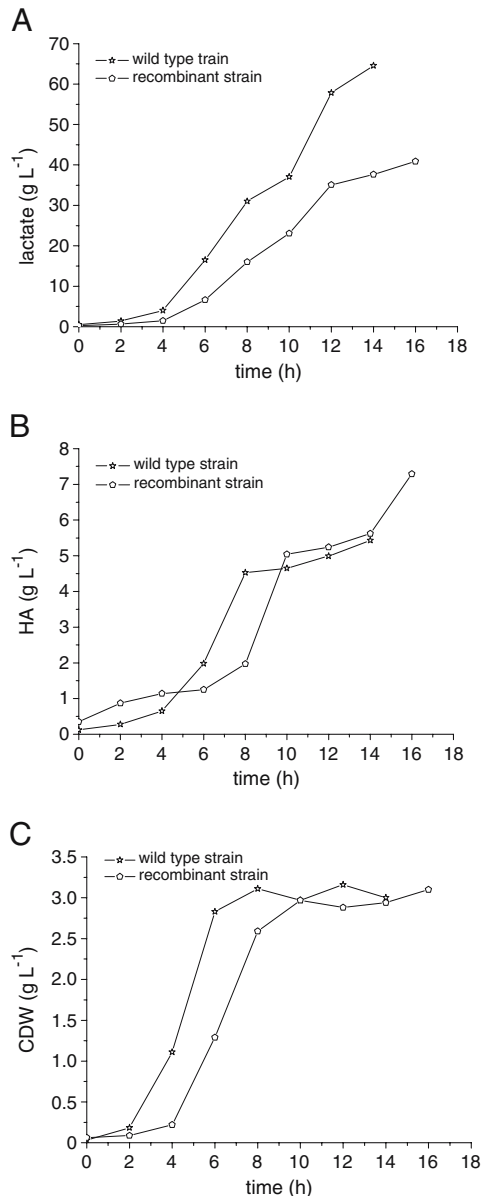


Fig. 2 Cell growth, lactic acid formation, and HA production by wild-type and recombinant *S. zooepidemicus* in a 7.5-L fermentor. **a** Lactic acid formation. **b** HA production. **c** Cell growth

Influence of *phbCAB* expression on the production of hyaluronic acid and lactic acid in fermentor cultures

S. zooepidemicus was cultured in sucrose (70 g L⁻¹) at 37°C for 14–16 h. The pH was controlled at approximately 7.0. The fermentation was terminated when the DO increased from zero to more than 50%. Compared with the wild-type strain, the recombinant strain took two more hours to complete the fermentation (Fig. 2). At 14 h, the wild type completed the fermentation cycle, producing 65 g L⁻¹ lactic acid and 5.4 g L⁻¹ HA. At the same time, the lactic acid and HA produced by the recombinant strain were 38 and 5.6 g L⁻¹, respectively. Compared with the wild-type strain, lactic acid formation was decreased by 42% under the same conditions. After 16 h, the recombinant strain completed the fermentation; it produced 41 g L⁻¹ lactic acid, significantly less than that produced by the wild type. The final HA concentration produced by the recombinant strain was 7.3 g L⁻¹, a 34% increase compared with that of the wild type. The cell densities of the two strains were approximately the same (Fig. 2).

Discussion

To express *phbCAB* in *S. zooepidemicus*, the gene was cloned and inserted under the *Spac* promoter. The activities of PhbA and PhbB in the recombinant strain were 3.13 and 1.23 U mg⁻¹, respectively. The enzyme activities in *E. coli* harboring pBHR69 were 3.25 and 1.27 U mg⁻¹, respectively (Chen et al. 2004). This indicated that PhbA and PhbB had prominent activities in the recombinant *S. zooepidemicus*. These results suggested that the genes had been successfully expressed in *S. zooepidemicus* (Table 1). NADPH can be transformed to NADP by the action of PhbB. Thus, the expression of *phbAB* could be sufficient to obtain the oxidation potential. Interestingly, no PhbC activity and no PHB accumulation were detected. Because *phbA* and *phbB* had been successfully transcribed and translated, *phbC*, the first gene under the *Spac* promoter, should also be immediately transcribed. To investigate the reasons for the lack of PhbC activity, *phaPQRBC* gene, including its own promoter from gram-positive *Bacillus megaterium*, was cloned and transformed into *S. zooepidemicus* (McCool and Cannon 1999). After coexpression of *phbA* from *R. eutropha* with *phaPQRBC* from *B. megaterium*, about 2% PHB was accumulated in *E. coli*, yet no PHB accumulation was observed in *S. zooepidemicus*. PhbA and PhbB are both intracellular proteins; however, PhbC is a membrane-bound protein. These results could suggest that *S. zooepidemicus* had a special mechanism for the insertion of membrane proteins. The action of PhbAB could provide oxidation potential for the cells. At the same time, acetyl-CoA was transformed to 3-hydroxybutyryl-CoA. This substance cannot be accumulated in cells. It should be consumed by certain metabolic pathways such as rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (Rehm et al. 2001). Because the genetic background of *S. zooepidemicus* is not very clear, more

research is needed to find out the metabolic flux of 3-hydroxybutyryl-CoA.

In shake flask cultivation, the pH could not be maintained constantly at about 7; it reached 4–5 during the stationary phase. Thus, the cell density was low (about 0.8 g L^{-1}), and only 0.6 g L^{-1} HA was produced (Table 2). This low concentration of HA did not create the high viscosity and low DO level that prevent cells from growing. Under these conditions, NAD could be regenerated mainly from two pathways. The first pathway is reoxidation via the NADH oxidase (NOX) of NADH, which catalyzes the four-electron reduction of molecular oxygen to water (Ross and Claiborne 1992). The second pathway is reoxidation of NADH via lactic acid dehydrogenase, which catalyzes the reduction of pyruvate to lactic acid. The former pathway also protects the cells from the damaging effects of oxygen by reducing it to water (Stanton and Sellwood 1999). These metabolic pathways are necessary for the survival of the aerotolerant *Streptococci*. When *phbCAB* gene is introduced into *S. zooepidemicus* as a NAD regeneration pathway, the two pathways should be able to influence each other. Under the oxygen-rich condition, the NOX pathway oxidizes a good part of the NADH (Chong and Nielsen 2003a,b). This could compensate for the consumption of oxidation potential related to the *phbCAB* pathway. However, due to the rapid reduction of pH from 7 to 4, cells grew very little; thus, the cell density and lactic acid production were both low in flask cultivation. Therefore, the effect of introducing the heterogenous NAD regeneration pathway into lactic acid and HA formation was not obvious (Table 2).

In the fermentor cultivation, the lag phase of the recombinant strain was 2 h longer than that of the wild type. This phenomenon also occurred in the fermentations of the strain harboring other plasmids. It could be attributed to the existence of antibiotics. However, there was no significant difference in the exponential and stationary phases between the two strains. The recombinant strain had a 2-h delay in ending the fermentation compared with the wild type. In the fermentor studies, lactic acid production by the recombinant strain was significantly reduced compared with that of the wild type (Fig. 2), as the viscosity generated by HA was much higher in a fermentor in which cells grew much better under constant pH. Under high viscosity, cells quickly entered an oxygen limitation stage ($\text{DO}=0$), which was nearly an anaerobic environment. The oxygen limitation led to a reduced activity of the NOX pathway. The NAD regeneration in the cells mainly relied on the lactic acid pathway: More than 75% of carbon source used was metabolized to lactic acid (Johns et al. 1994; David and Michael 1997). Lactic acid formation is sensitive to the cellular oxidation/reduction level (Chong and Nielsen 2003a,b). Overexpressing NADH oxidase gene or increasing the DO level can decrease lactic acid production (Chong and Nielsen 2003a,b; Gao 1999). In fact, lactate dehydrogenase is sensitive to cellular NAD/NADH level (Garrigues et al. 1997). In the fermentor cultivation pro-

cess, lactate formation by the recombinant strain was sharply decreased. This phenomenon provided strong evidence for the introduction of *phbCAB* genes that had increased the cellular NAD/NADH level. The expression of *phbCAB* gene in *S. zooepidemicus* provided a heterogenous NAD regeneration pathway for the cells, which could have a significant influence on lactic acid formation (Fig. 2a). Acting as a pathway for the generation of cellular oxidation potential, the *phbCAB* pathway reduced the pressure of the lactic acid pathway. At the same time, the decreased carbon flux to lactic acid allowed more carbon flux to go to other metabolic pathways; the increase of HA production was supposed to be one of the effects of the changing carbon flux (Fig. 2b).

In this study, for the first time, PHB synthesis genes (*phbCAB*) were used to increase the cellular oxidation potential in the HA-producing bacterium *S. zooepidemicus*. This recombinant pathway significantly decreased the flux to the original NADH reoxidation pathway, the lactic acid pathway of *S. zooepidemicus*. Compared with other NAD regeneration pathways, such as glutamate dehydrogenase, yeast alcohol dehydrogenase, lactic acid dehydrogenase, and NADH oxidase (Geueke et al. 2003), there are no harmful by-products formed. This indicates that the PHB synthesis pathway could be employed as a global regulator to adjust the cellular oxidation/reduction potential.

PHAs have been investigated as a final product, and various methods were used to regulate the PHA synthesis. This is the first study demonstrating that PHA synthesis related to energy and carbon metabolism could be employed as a pathway to regulate other cellular metabolism and possibly to regulate the production of other desired metabolic products.

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