Metabolic Engineering of Escherichia coli for Poly(3-hydroxybutyrate) Production under Microaerobic Condition

Xiao-Xing Wei, 1 Wei-Tao Zheng, 2 Xue Hou, 1 Jian Liang, 3 and Zheng-Jun Li 2

1Department of Basic Medicine, Medical College of Qinghai University, Xining 81006, China
2Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China
3College of Eco-Environmental Engineering of Qinghai University, Xining 810016, China

Correspondence should be addressed to Zheng-Jun Li; lizj@mail.buct.edu.cn

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The alcohol dehydrogenase promoter PadhE and mixed acid fermentation pathway deficient mutants of Escherichia coli were employed to produce poly(3-hydroxybutyrate) (P3HB) under microaerobic condition. The E. coli mutant with ackA-pta, poxB, ldhA, and adhE deletions accumulated 0.67 g/L P3HB, up to 78.84% of cell dry weight in tube cultivation. The deletion of pyruvate formate-lyase gene pflB drastically decreased P3HB production and P3HB content to 0.09 g/L and 24.44%, respectively. Overexpressing pflB via the plasmid in its knocked out mutant restored cell growth and P3HB accumulation, indicating the importance of the pyruvate formate-lyase in microaerobic carbon metabolism. The engineered E. coli BWapld (pWYC09) produced 5.00 g/L P3HB from 16.50 g/L glucose in 24 h batch fermentation, and P3HB production yield from glucose was 0.30 g/g, which reached up to 63% of maximal theoretical yield.

1. Introduction

Poly(3-hydroxybutyrate) (P3HB) is a polyester of 3-hydroxybutyrate that is synthesized by a variety of microorganisms as intracellular carbon and energy storages under unbalanced nutrients conditions [1]. The mechanical properties of P3HB are similar to those of petro derived polymers such as polypropylene, which makes it a promising substitution of common plastics [2]. Moreover, P3HB can be obtained from renewable carbon sources and easily degraded into CO2 and H2O when discarded in natural environments. Thus, microbial production of P3HB via fermentation has attracted extensive attentions during the past few decades [3].

Although considerable efforts have been devoted to decreasing the production cost of P3HB to make it economically competitive, the present price of P3HB is still not feasible to replace traditional petro-based plastics. Generally, P3HB was mainly produced from renewable carbon sources through aerobic fermentation [4, 5]. Given that the feedstock especially carbon source accounts up to a large portion of the production cost, low cost substrates such as crude glycerol and agricultural wastes have been explored for P3HB production [4, 6, 7]. On the other hand, aerobic fermentation process can be high demand of stir and air supply and contribute further energy cost besides feedstock. Microaerobic process could alleviate the energy requirement for fermentation by simplifying the bioreactor design and control strategies. Considering the potential advantages, P3HB production under microaerobic conditions has been investigated [8]. However, the P3HB content obtained under fed-batch microaerobic cultures was 51%, lower than that produced by aerobic fermentation [9]. Low polymer content decreases the purification efficiency and results in extra expenses in downstream process.

To achieve high P3HB content under microaerobic condition, the native promoter of P3HB biosynthesis operon pha-CAB from Ralstonia eutropha was changed to the anaerobic alcohol dehydrogenase promoter PadhE, which was reported
to be upregulated under oxygen limited condition. As a result, P3HB content was improved from 30% to 48% of cell dry weight [10]. Moreover, E. coli undertakes mixed acid fermentation pathway, producing lactate, succinate, acetate, formate, and ethanol under oxygen limited conditions, which decrease the carbon flux into P3HB accumulation. E. coli mutant with deletions of ackA-pta, poxB, ldhA, and adhE was constructed and doubled the cell dry weight and improved P3HB production by 3.5-folds compared to the control [11].

In this paper, we managed to further increase the microaerobic P3HB production by employing the PphaE promoter to drive the expression of phaCAB operon in E. coli mutants with defected mixed acid fermentation pathway. Moreover, the pyruvate formate-lyase, which catalyzes the coenzyme A-dependent, nonoxidative cleavage of pyruvate to acetyl-CoA and formate under anaerobic or microaerobic conditions, was overexpressed to investigate its effect on cell growth and P3HB accumulation (Figure 1).

2. Materials and Methods

2.1. Bacterial Strains and Plasmids Construction. Bacterial strains, plasmids, and primers used in this study were listed in Table 1. E. coli BW25113 mutants with serial deletions of ackA-pta, poxB, ldhA, adhE, and pflB were constructed to eliminate the mixed acid fermentation pathway. Plasmid pWYC09 was constructed to express phaCAB operon from R. eutropha with promoter PphaE. The pyruvate decarboxylase promoter of Zymomonas mobilis was amplified with primers pdcF/pdcR and then ligated into the XhoI/NdeI site of pBBRIMCS-2 to construct pMCS2pdc. pflB was amplified from E. coli BW25113 genome with primers pflF/pflR and inserted into the NdeI/EcoRI site of pMCS2pdc to generate pMCS2pflB.

2.2. Culture Conditions. For tube cultivation, one percent seed culture was inoculated into a 250 mL sealed tube completely filled with Luria-Bertani (LB) medium supplemented with 10 g/L glucose and then maintained at 37°C for 48 h as static cultures. For 5.5 liter bioreactor cultivation, 50 mL seed culture was transferred to the bioreactor containing 3 L LB medium supplemented with 20 g/L glucose. The pH was maintained at 7.0 via automatic addition of 5 M sodium hydroxide solution. Batch cultures were performed without air supply; the agitation was set at 75 rpm to prevent biomass sedimentation and maintain heat transfer and substrate exchange.

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**Table 1:** Strains, plasmids, and primers used in this study.

| Strain/Plasmid | Description | Reference |
|----------------|-------------|-----------|
| E. coli BW25113 | lacIq rrrBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78 | [11] |
| BWa | BW25113 ΔackA-pta | [11] |
| BWap | BW25113 ΔackA-pta, ΔpoxB | [11] |
| BWapl | BW25113 ΔackA-pta, ΔpoxB, ΔldhA | [11] |
| BWapld | BW25113 ΔackA-pta, ΔpoxB, ΔldhA, ΔadhE | [11] |
| BWapldf | BW25113 ΔackA-pta, ΔpoxB, ΔldhA, ΔadhE, ΔpflB | [11] |
| Plasmids | pWYC09 pBluescriptIISK − derivatives containing phaCAB from R. eutropha with promoter PhphaE, AmpR | [10] |
| | pBBRIMCS-2 Broad-host-range plasmid, KanR | [10] |
| | pMCS2pdc Promoter PphaE inserted into pBBRIMCS-2 This study | |
| | pMCS2pflB pflB inserted into pMCS2pdc This study | |
| Primers | pdcF 5′-ATACTCGAGTTACGCTCATGATCGCGGCATGTC | |
| | pdcR 5′-CCCCATATGTTACTCCATATATTCAAAAC | |
| | pfIF 5′-GCTAGGGCATATGGTCCGAGCTTAAATGAAA | |
| | pfIR 5′-CCGAAATTTCATACATAGATTTGAAGGT | |

All oligonucleotides were synthesized by AuGCT Biotechnology (Beijing, China). Restriction endonuclease digestion sites were underlined.
Table 2: P3HB accumulation and by-product formation of E. coli BW25113 and its five mutants harboring P\textsubscript{adhE} controlled phaCAB operon.

| E. coli          | CDW (g/L) | P3HB content (%) | Glucose consumed (g/L) | Succinate (g/L) | Lactate (g/L) | Formate (g/L) | Acetate (g/L) | Ethanol (g/L) |
|-----------------|-----------|------------------|------------------------|----------------|--------------|--------------|--------------|--------------|
| BW25113         | 1.24 ± 0.08 | 44.85 ± 1.46     | 7.59 ± 0.09           | 0.34 ± 0.04    | 1.21 ± 0.12  | 0.06 ± 0.01  | 1.59 ± 0.08  | 0.25 ± 0.04  |
| BWa             | 0.73 ± 0.10 | 68.31 ± 4.86     | 5.31 ± 0.16           | 0.37 ± 0.06    | 1.47 ± 0.26  | 0.04 ± 0.01  | 0.35 ± 0.02  | 0.19 ± 0.02  |
| BWap            | 0.57 ± 0.06 | 70.69 ± 5.35     | 4.89 ± 0.24           | 0.28 ± 0.06    | 1.41 ± 0.16  | 0.03 ± 0.01  | 0.26 ± 0.06  | 0.21 ± 0.04  |
| BWapld          | 1.33 ± 0.08 | 73.58 ± 4.72     | 6.26 ± 0.18           | 0.58 ± 0.08    | ND           | 0.06 ± 0.02  | 0.45 ± 0.04  | 0.53 ± 0.06  |
| BWapldf         | 0.85 ± 0.04 | 78.84 ± 3.27     | 5.14 ± 0.23           | 0.32 ± 0.04    | ND           | ND           | 0.31 ± 0.02  | ND           |
| BWapldf         | 0.36 ± 0.03 | 24.11 ± 3.95     | 2.32 ± 0.08           | 0.18 ± 0.02    | ND           | ND           | 0.20 ± 0.05  | ND           |

The recombinants harboring pWYC09 were cultivated in 250 mL sealed tubes completely filled with LB medium supplemented with 10 g/L glucose at 37°C for 48 h. CDW: cell dry weight. ND: not detected. Data shown are the average and standard deviation of three parallel experiments.

2.3. Analytical Methods. Cells were harvested by centrifugation at 8,000 × g and 4°C for 10 min. The cell pellets were washed twice with distilled water and then lyophilized for 12 h for cell dry weight (CDW) assay. Lyophilized cells were subjected to methanolysis at 100°C for 4 h in the presence of 3% (v/v) H\textsubscript{2}SO\textsubscript{4} and then assayed with a gas chromatograph (GC) to measure the P3HB content. For glucose and by-products lactate, succinate, acetate, formate, and ethanol measurements, the supernatant of cell cultures was filtered through a 0.2 μm syringe filter and then analyzed by high-performance liquid chromatography (HPLC) equipped with an ion exchange column (Aminex HPX-87H, 300 × 7.8 mm) and a refractive index detector.

3. Results and Discussion

3.1. P\textsubscript{adhE} Controlled P3HB Production in E. coli Mixed Acid Fermentation Mutants. The alcohol dehydrogenase promoter P\textsubscript{adhE} has been demonstrated to improve the expression level of phaCAB operon under microaerobic condition [10]. Moreover, the disruption of mixed acid fermentation pathway was proved to be another effective strategy to increase P3HB accumulation [11]. To investigate the possibility of the synergistic effect, plasmid pWYC09 carrying P\textsubscript{adhE} controlled phaCAB was transformed into the wild type E. coli and a series of mixed acid fermentation mutants BWa, BWap, BWapld, BWapldf, and BWapld (Table 1). The recombinant strains were cultivated in LB medium supplemented with glucose to produce P3HB under microaerobic tubes. The mixed acid fermentation mutants showed higher P3HB content compared to that of wild type except for the pflB mutant, and P3HB accumulated up to 78.84% of cell dry weight in the quadruple ΔackA-pta, ΔpoxB, ΔldhA, and ΔadhE deletion mutant BWapld, which produced the fewest amount of mixed acid (Table 2). Compared to the strains containing phaCAB operon with its native promoter, P\textsubscript{adhE} driven operon led to much higher P3HB production [11].

When pflB was knocked out, both cell growth and P3HB accumulation decreased significantly (Table 2). It was reported that the expression of pyruvate formate-lyase was induced to generate acetyl-CoA and formate from pyruvate when E. coli was cultivated with limited oxygen. Therefore, the deletion of pflB may cause the disruption of glucose metabolism and led to much lower cell biomass and P3HB accumulation. The profile of by-product formation was similar to the previous report [11]. Lactate production was totally eliminated by deleting ldhA, and only minute amount of acetate and succinate was detected in BWapld and BWapldf (Table 2).

3.2. Effect of pflB Overexpression on Cell Growth, P3HB Accumulation, and By-Product Formation. Considering the importance of pflB in glucose metabolism under oxygen limited condition, plasmid pMCS2pflB was constructed to constitutively expressing pflB and cotransformed with pWYC09 into E. coli to study its influence on cell metabolism profile. The plasmid carrying pflB overexpression restored the cell growth and P3HB production completely in pflB deletion mutant BWapldf. Cell dry weight was increased from 0.36 g/L to 0.70 g/L, and P3HB content was increased from 24.44% to 44.57%, which confirmed the crucial role of pflB during the microaerobic P3HB production (Table 3). However, pflB overexpression did not affect the cell growth or P3HB accumulation significantly in other mutants; only a slight improvement in terms of the CDW and P3HB content was observed in BWapld mutant. Compared to those of the control without pflB overexpression, BWapld harboring pWYC09 and pMCS2pflB grew up to 1.16 g/L CDW and accumulated 84.79% of P3HB, exhibiting 37% and 8% increase, respectively (Table 3). These results indicate that the expression of pflB from its native genomic copy could satisfy the requirement of E. coli cells for most of cases of experimental condition, and its expression was proved be of crucial importance to oxygen limited glucose metabolism. Furthermore, no large amount of formate production was observed in all pflB overexpressing strains, probably due to the expression of formate dehydrogenase, converting formate to CO\textsubscript{2} and H\textsubscript{2}O when formate amount exceeds the tolerance of E. coli [12].

3.3. Growth and P3HB Production by E. coli BWapld Mutants in a 5.5-Liter Fermenter. The recombinant E. coli BWapld (pWYC09) and BWapld (pWYC09 + pMCS2pflB) were cultivated in oxygen limited 5.5 liter bioreactor to investigate cell growth and P3HB production profile. After 24 h cultivation, the recombinant BWapld (pWYC09) consumed 16.50 g/L glucose and produced 5.00 g/L P3HB; the P3HB content reached 73.58% of cell dry weight. The by-product formation
Table 3: Influence of pflB overexpression on cell growth, P3HB accumulation, and by-product formation.

| E. coli | CDW (g/L) | P3HB content (%) | Glucose consumed (g/L) | Succinate (g/L) | Lactate (g/L) | Formate (g/L) | Acetate (g/L) | Ethanol (g/L) |
|---------|-----------|------------------|-----------------------|----------------|--------------|--------------|--------------|--------------|
| BW25113 | 0.85 ± 0.03 | 38.44 ± 5.91 | 6.95 ± 0.17 | 0.68 ± 0.03 | 0.97 ± 0.09 | 0.23 ± 0.02 | 1.68 ± 0.01 | 0.45 ± 0.06 |
| BWa     | 0.58 ± 0.08 | 71.58 ± 6.93 | 5.31 ± 0.13 | 0.72 ± 0.06 | 1.29 ± 0.12 | 0.20 ± 0.01 | 0.64 ± 0.06 | 0.29 ± 0.04 |
| BWap    | 0.64 ± 0.02 | 66.22 ± 4.68 | 4.89 ± 0.12 | 0.64 ± 0.01 | 1.16 ± 0.08 | 0.18 ± 0.02 | 0.24 ± 0.07 | 0.25 ± 0.03 |
| BWapl   | 1.12 ± 0.07 | 76.47 ± 2.98 | 5.36 ± 0.26 | 0.84 ± 0.02 | ND | 0.11 ± 0.04 | 0.29 ± 0.04 | 0.38 ± 0.05 |
| BWapldf | 0.70 ± 0.06 | 44.57 ± 4.02 | 3.78 ± 0.26 | 0.73 ± 0.02 | ND | 0.15 ± 0.04 | 0.16 ± 0.05 | ND |

The recombinants harboring pWYC09 and pMCS2pflB were cultivated in 250 mL sealed tubes completely filled with LB medium supplemented with 10 g/L glucose at 37°C for 48 h. CDW: cell dry weight. ND: not detected. Data shown are the average and standard deviation of three parallel experiments.

Figure 2: Cell dry weight (CDW), P3HB content (%), glucose consumption, and mixed acid production profile of E. coli BWapld harboring P_adhE controlled phaCAB operon without (a) or with (b) pflB overexpression in 5.5 liter bioreactor under microaerobic condition.

was maintained at low level during the process, and finally 0.23 g/L succinate, 0.86 g/L acetate, 0.17 g/L formate, and 0.03 g/L ethanol were detected (Figure 2(a)). When pflB was overexpressed, the final CDW and P3HB content were 5.88 g/L and 84.43%, respectively, accompanied with a total amount of 1.72 g/L mixed acid (Figure 2(b)). Previously, the wild type strain BW25113 harboring the same plasmid produced 6.72 g/L CDW with 44.53% P3HB under the same condition. In this study, with the disruption of mixed acid fermentation pathways, the recombinant accumulated P3HB up to 73.58% of cell dry weight, and overexpressing pflB helped to further improve P3HB content to 84.43%, which will facilitate downstream biopolymer purification processes.

In terms of byproduct formation and P3HB production yield, the wild type strain BW25113 produced 13.60 g/L mixed acids, and pta mutant JW2294 generated 16.60 g/L mixed acids (Table 4). In this study, the mixed acid fermentation pathway mutant BWapld only produced 1.29 g/L mixed acids. As a result, the P3HB production yield from glucose was significantly increased. The wild type obtained 0.10 g P3HB/g glucose, and the pta mutant showed a 70% increase to 0.17 g/g. BWapld mutant reached up to 0.30 g/g, which was 2 times higher than that obtained from the wild type (Table 4).

The maximal theoretical yield of P3HB from glucose is 0.48 g P3HB/g glucose. Previously, the arcA mutant showed 46% of maximal yield under optimized microaerobic fermentation condition [9]. The recombinant constructed in this study reached up to 63% of maximal yield. Hence, the engineered E. coli constructed here showed superior ability for microaerobic P3HB production compared to previous strains. Further optimization of different degrees of oxygen limitation in bioreactor batch cultivations should be performed to obtain much higher P3HB production titer and productivity. Since the overall redox balance is important to P3HB biosynthesis and the metabolic response [13], P3HB production with other substrates with different oxidation states of the carbon atoms such as gluconate and glycerol could be another choice for improvements under microaerobic condition.

4. Conclusion

The alcohol dehydrogenase promoter and mixed acid fermentation mutants were combined to improve P3HB production under microaerobic condition. The deletion of pflB gene significantly decreased cell biomass and P3HB production...
The optimization of glucose was 0.30 g/g, up to 63% of maximal theoretical bioreactor fermentation. The P3HB production yield from 5.00 g/L P3HB, up to 73.58% of cell dry weight in 5.5 liter engineered strain BWapld harboring pWYC09 accumulated of pyruvate formate-lyase for microaerobic metabolism. The growth and P3HB accumulation, indicating the importance fermentation mutants was proved to be effective for improving microaerobically P3HB production.

Table 4: Summary of engineered E. coli for improving P3HB production under microaerobic condition.

| E. coli            | Description                                      | Cell dry weight (g/L) | P3HB content (%) | P3HB concentration (g/L) | Mixed acid (g/L) | Yield (g P3HB/g glucose) |
|--------------------|--------------------------------------------------|-----------------------|------------------|--------------------------|-----------------|--------------------------|
| BW25113 Wild type  |                                                  | 6.72                  | 44.53            | 3.06                     | 13.60           | 0.10                     |
| JW2294 pta knockout|                                                  | 7.75                  | 64.31            | 4.98                     | 16.60           | 0.17                     |
| BWapld pflB        | Mixed acid fermentation deficient                | 6.79                  | 73.58            | 5.00                     | 1.29            | 0.30                     |
| BWapld (pMCS2pflB) | Mixed acid fermentation deficient and pflB overexpression | 5.88                  | 84.43            | 4.96                     | 1.72            | 0.27                     |

All E. coli strains carried plasmid pWYC09, which harbored phaCAB operon under the control of P_adaE. Mixed acid referred the total amount of succinate, formate, acetate, and ethanol.

and overexpressing pflB from the plasmid restored the cell growth and P3HB accumulation, indicating the importance of pyruvate formate-lyase for microaerobic metabolism. The engineered strain BWapld harboring pWYC09 accumulated 5.00 g/L P3HB, up to 73.58% of cell dry weight in 5.5 liter bioreactor fermentation. The P3HB production yield from glucose was 0.30 g/g, up to 63% of maximal theoretical yield. The optimization of phaCAB expression in mixed acid fermentation mutants was proved to be effective for improving microaerobically P3HB production.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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