Enhanced Functional Expression of the Polyhydroxyalkanoate Synthase Gene from Cupriavidus Necator A-04 Using a Cold-Shock Promoter for Efficient Poly(3-Hydroxybutyrate) Production in Escherichia Coli

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Abstract

**Background:** The present study attempted to increase PHB production by improving the functional expression of the PhaC gene using various types of promoters, and the effects on PhaC activity in terms of PHB productivity, yield coefficient ($Y_{P/S}$) and molecular weights were investigated.

**Results:** Here, the PHB biosynthesis operon of *Cupriavidus necator* A-04, isolated in Thailand with a high degree of 16S rRNA sequence similarity with *C. necator* H16, was subcloned into pGEX-6P-1, pColdI, pColdTF, pBAD/Thio-TOPO and pUC19 (constitutive expression) and transformed into *E. coli* JM109. To alter the expression of *phaCAB* biosynthesis genes, we optimized parameters in flask experiments to obtain high expression of soluble PhaC$_{A-04}$ protein with high $Y_{P/S}$ and PHB productivity. pColdTF-phaCAB$_{A-04}$-expressing *E. coli* produced 2.5 ± 0.1 g/L (90.6±4.3%) PHB in 24 h, similar to pColdI-phaCAB$_{A-04}$-expressing *E. coli*. The amounts of phaC protein and PHB produced from pColdTF-phaCAB$_{A-04}$ and pColdI-phaCAB$_{A-04}$ were significantly higher than those from other promoters. Cultivation in a 5-L fermenter led to PHB production of 7.9±0.7 g/L with 90.0±2.3% PHB content in the cell dry mass (DCM), a $Y_{P/S}$ value of 0.38 g PHB/g glucose and a productivity of 0.26 g PHB/(L⋅h) using pColdTF-phaCAB$_{A-04}$. The PHB from pColdTF-phaCAB$_{A-04}$ had $M_W$ 5.79 $\times 10^5$ Da, $M_N$ 1.86 $\times 10^5$ Da and PDI 3.11 and the film exhibited high transparency, Young’s modulus and tensile strength, possibly due to the TF chaperones. Interestingly, when pColdI-phaCAB$_{A-04}$-expressing *E. coli* was used to produce PHB from crude glycerol and compared with constitutive pUC19-nativeP-phaCAB$_{A-04}$-expressing *E. coli*, the amounts of PHB were similar, but $M_W$ 1.1 $\times 10^6$ Da, $M_N$ 2.6 $\times 10^5$ Da and PDI 4.1 were obtained from constitutive pUC19-nativeP-phaCAB$_{A-04}$-expressing *E. coli*, indicating that slow and low expression could prolong and maintain phaC polymerization activity.

**Conclusions:** The cspA promoter in a cold-inducible vector can improve PhaC$_{A-04}$ expression levels, and TF chaperones show obvious effects on enhancing PhaC$_{A-04}$ solubility. The high level of phaC$_{A-04}$ resulted in a high amount of PHB, but the chain termination reaction of PhaC polymerization occurred faster than that with the retarded and low expression of phaC$_{A-04}$ by the constitutive promoter pUC19, which in turn resulted in a low amount of PHB with a high molecular weight.

Background

The global environmental concern regarding microplastics in the marine environment as contaminants with significant impacts on animal and human health has led to a call for national and international policies from more than 60 countries to ban or place a levy on single-use plastics [1–4]. Renowned global companies have also integrated regulations and policies to ban plastic bottle cap seals, plastic water bottles, straws and other single-use packaging into their green marketing and corporate social responsibility policies. Bioplastics are becoming a popular alternative to single-use plastics to reduce the amount of microplastic waste. Recently, the annual production of bioplastics was approximately one percent of the total 360 million tons of plastics. However, as the market for bioplastics is growing and the demand for bioplastics is rising, European Bioplastics reported that the global bioplastic production capacity will increase from 2.11 million tons in 2019 to approximately 2.43 million tons in 2024 [5]. Among the various types of bioplastics, polyhydroxyalkanoates (PHAs) are an important biodegradable polymer family, as they are one hundred percent biobased and fully biodegradable in all environments, especially marine (ASTM 7081) and fresh water environments [6, 7]. Notably, their variety of monomeric compositions offers a wide array of thermal, physical and mechanical properties that strongly rely on PHA-producing strains and carbon sources.

To obtain both the environmental and economic benefits of PHAs over synthetic plastics and other bioplastics, microorganisms that exhibit efficient PHA production from inexpensive and renewable carbon sources are urgently required to develop a low-cost approach. Microbial cells typically accumulate polyhydroxybutyrate
(PHB), the first PHA discovered since 1927 by Maurice Lemoigneni of Institut Pasteur in France [8], at approximately 30–50% of the dry cell mass (DCM). The most well-known industrial PHA producer, Cupriavidus necator H16 (formerly known as Alcaligenes eutrophus and Ralstonia eutropha), is capable of accumulating PHB at over 80% of the DCM. PHA accumulation is tightly regulated by imbalanced growth conditions with excess carbon but limited nitrogen [9]. One of the major limitations in the production of PHAs in wild-type strains has been intracellular polymer degradation caused by endogenous PHA depolymerases, which is different from the behavior of exogenous PHA depolymerases [10]. Therefore, intracellular PHAs are often spontaneously degraded during cultivation when the bacteria require carbon, resulting in low PHA content and a wide range of molecular weight distributions in wild-type strains. Thus, many recombinant strains have been developed by metabolic engineering to obtain a high yield of PHB and a molecular weight that is high enough for polymer processing [11–16]. Ordinarily, the PHB biosynthesis pathway begins with acetyl-CoA and requires 3 major enzymes, namely, 3-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC), and these 3 genes are sufficient for the production of PHB in non-PHA-producing bacteria at more than 90% of the DCM when heterologously expressed in Escherichia coli [17]. It has been reported that PhaC plays a key role in obtaining the polymeric form, resulting in a high level and high molecular weight of PHB [14, 16].

To date, PHA synthases have been categorized into four major classes based on their sequence, substrate specificity, and subunit composition [18, 19]. Class I and Class II PHA synthases consist of the PhaC subunit, which is believed to be a homodimer. On the other hand, Class III and IV PHA synthases are heteroclusters comprised of PhaC-PhaE subunits and PhaC-PhaR subunits, respectively. In addition, Class I, III, and IV PHA synthases preferentially polymerize short-chain-length (SCL) monomers comprised of C3-C5 carbon chain lengths, whereas Class II PHA synthases specifically polymerize medium-chain-length (MCL) monomers in the C6-C14 chain length range. It was reported that PhaC derived from C. necator H16 (PhaC_{H16}) is a Class I PhaC and is one of the most widely studied PHA synthases. It has a molecular weight of approximately 64 kDa (589 amino acids) and is located as the first gene in the PHA biosynthetic operon, followed by PhaA and PhaB [20, 21]. It was demonstrated that the weight-average molecular weight (M_w) of PHB synthesized by wild-type bacteria is generally in the range of 0.1–2.0 × 10^6 Da. Because Escherichia coli does not contain PHA depolymerase, ultrahigh-molecular-weight PHB, which has a defined M_w more than 3.0 × 10^6 Da and is much larger than the PHB produced by the wild-type strain, can be obtained and used for the development of high-strength fibers and films [22]. Unfortunately, no crystal structure exists for any PHA synthase enzyme because this enzyme tends to form inclusion bodies when overexpressed in a bacterial host or tends to aggregate during purification [23]. When recombinant PhaC_{H16} was overexpressed in E. coli, most of the protein formed insoluble inclusion bodies due to its low aqueous solubility [24–27]. To feasibly achieve industrial-scale production, PhaC would need to be produced in large quantities and its solubility would need to be improved [23]. There have been many reports that have attempted to resolve the problem mentioned above, including by modulating the concentration of the PhaC protein by varying the chemical inducer quantities [28]; expressing the protein at a reduced temperature (30 °C) [23]; fusing the PhaC protein with a glutathione S-transferase (GST) tag, which is a hydrophilic tag, to improve its solubility [29]; and coexpressing the protein with chaperones to obtain high total quantities of enzyme and a larger proportion in the soluble fraction than obtained without chaperones. However, coexpression of the GroEL/GroES system with the PHA production operon resulted in the production of polymers with reduced molecular weights [23].

In a previous study, we reported the generation of the C. necator strain A-04, possessing 99.78% 16S RNA sequence similarity with C. necator H16 but differing in PHA production ability [30]. Designed using the gene walk technique, the PHA biosynthesis operon of C. necator strain A-04 consisted of three genes, encoding acetyl-CoA acetyltransferase (phaA_{A−04}, 1182 bp, 40.6 kDa, accession no. FJ897461), acetoacetyl-CoA reductase (phaB_{A−04}, 741 bp, 26.4 kDa, accession no. FJ897462) and PHB synthase (phaC_{A−04}, 1770 bp, 64.3 kDa, accession no. FJ897463). Sequence analysis of the phaA_{A−04}, phaB_{A−04} and phaC_{A−04} genes revealed that phaC_{A−04} was 99% similar to phaC_{H16} from C. necator H16. The difference was in the amino acid residue situated at position 122, which in phaC_{A−04} was proline but in C. necator H16 was leucine. The total amino acid sequences of phaA_{A−04} and phaB_{A−04} were 100% matched with those of C. necator H16 (data not shown, manuscript in preparation). Notably, C. necator strain A-04 prefers fructose over glucose as a carbon source,
accumulating PHB at 78% of the DCM under a C/N ratio of 200, whereas it could incorporate a high mole fraction of monomeric 4-hydroxybutyrate monomeric into the poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] copolymer under a C/N ratio of 20 [31] as well as the poly(3-hydroxybutyrate-co-3-hydroxyvurate-co-4-hydroxybutyrate) [P(3HB-co-3HV-co-4HB)] terpolymer [32]. Thus, in this study, the PHA biosynthesis operon of C. necator strain A-04 was amplified via PCR; cloned into pGEX-6P-1 (tac promoter, isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible vector, N-terminal GST fusion protein), pColdI (cspA promoter, cold- and IPTG-inducible vector, N-terminal 6His-fusion protein), pColdTF (cspA promoter, cold- and IPTG-inducible vector, trigger factor (TF) chaperone, N-terminal 6His-fusion protein), pBAD/Thio-TOPO (araBAD promoter, arabinose-inducible vector, N-terminal thioredoxin fusion protein and C-terminal 6His-fusion protein) and pUC19 (control strain, constitutive expression, phaCAB A−04 biosynthesis genes with native promoter of phaC A−04); and transformed into E. coli JM109. In this study, we examined the effect of phaC A−04 overexpression on PHB production in recombinant E. coli with respect to cell growth, glucose consumption, PHB production, and kinetic parameters in conditions ranging from flask culture to a 5-L fermenter. Furthermore, the produced PHB was subjected to molecular weight determination, thermal analysis and mechanical property measurement.

**Results**

**Effect of the growth phase on the production of PhaC A−04 and PHB by the conventional induction method**

To optimize the conditions for heterologous expression of phaCAB A−04 biosynthesis genes, after the pColdI-phaCAB A−04 and pColdTF-phaCAB A−04 vectors were transformed into E. coli JM109, expression was induced with 0.5 mM IPTG (final concentration) at different growth phases by varying OD600 based on cultivation time: 0.5 (2 h, early exponential phase), 1.3 (4 h, middle exponential phase), 2.1 (6 h, late exponential phase) and 2.4 (10 h, stationary phase). Concurrently, the temperature was shifted from 37 °C to 15 °C for 24 h. Figure 2 shows the effect of the growth phase for gene induction on the DCM (g/L), PHB content (% w/w) and levels of insoluble and soluble PhaC A−04 protein, comparing E. coli JM109 (pColdI-phaCAB A−04) and E. coli JM109 (pColdTF-phaCAB A−04). The PhaC A−04 protein was detected by western blot analysis using an anti-His tag antibody as the primary antibody. A band appeared in the western blot at the position corresponding to that of the His-tagged phaC A−04 protein (67 kDa) for pColdI-phaCAB A−04 and the fusion protein of His-tagged phaC A−04 and TF at 115 kDa. By varying the time courses of the growth phase, His-tagged PhaC A−04 and the His-tagged phaC A−04-TF fusion protein were successfully expressed, with the highest amount of total phaC A−04 protein obtained when the phaCAB A−04 operon was induced at an OD600 of 0.5 (2 h, early exponential phase). The content of soluble PhaC A−04-TF fusion protein (Fig. 2B, lane 3) in the sample after IPTG induction at an OD600 of 0.5 was much higher than that of the phaC A−04 protein alone from pColdI-phaCAB A−04 (Fig. 2A, lane 3), suggesting that the TF chaperone facilitates the expression of highly soluble protein in E. coli JM109. Moreover, significant proteolysis of the PhaC A−04 protein occurred with pColdI-phaCAB A−04 when cells produced a large amount of insoluble PhaC A−04 during the exponential phase, resulting in smeared bands of degraded insoluble proteins. On the other hand, proteolysis of the PhaC A−04 protein was not observed with pColdTF-phaCAB A−04 when cells were induced with 0.5 mM IPTG at an OD600 of 0.5. Notably, the highest amount of soluble PhaC A−04 and TF fusion protein was produced only at an OD600 of 0.5 and was not detected in other growth phases. It was observed that the TF protein could enhance PhaC A−04 solubility and prevent PhaC A−04 degradation. Functional PhaC A−04 protein production was confirmed by determining the amount of PHB produced; however, the value was only 46.57% w/w with a productivity of 0.03 ± 0.01 g/(L·h).

**Development of a short-induction method and effect of IPTG concentration on PHB productivity**

Next, a short-induction method was investigated in this study with the aim of accelerating growth and PHB production and attaining higher productivity than that afforded by the conventional induction method. The optimization was carried out based on one-factor-at-a-time experiments by varying one factor of interest while
keeping other factors constant [33]. First, conditions were optimized by varying the OD$_{600}$ based on cultivation time (0.5, 1.3, 2.1 and 2.4 h) and inducing expression with 0.5 mM IPTG at 15 °C for 30 min. Then, the temperature was shifted from 15 °C to 37 °C for 24 h to enhance growth and PHB production. The effect of the growth phase (OD$_{600}$) on DCM (g/L) and PHB content (% w/w) is illustrated in Fig. 3. Again, it was clearly observed that cells of both E. coli JM109 (pColdI-phaCAB$_{A-04}$) and E. coli JM109 (pColdTF-phaCAB$_{A-04}$) in the 2-h early exponential phase (OD$_{600}$ of 0.5) exhibited higher DCM and PHB production than those in other growth phases. After induction with 0.5 mM IPTG at 15 °C for 30 min and cultivation at 37 °C for 24 h, E. coli JM109 (pColdI-phaCAB$_{A-04}$) attained 4.5 ± 0.1 g/L DCM, 3.9 ± 0.1 g/L PHB and 85.90 ± 2.6% (w/w) PHB content with a productivity of 0.16 g PHB/(L⋅h), whereas E. coli JM109 (pColdTF-phaCAB$_{A-04}$) attained 3.5 ± 0.1 g/L DCM, 2.7 ± 0.1 g/L PHB and 75.90 ± 2.8% (w/w) PHB content with a productivity of 0.11 g PHB/(L⋅h). Thus, the short-induction method enhanced the PHB content and productivity more than the conventional method.

Next, an OD$_{600}$ of 0.5 was used to investigate the optimal concentration of IPTG (0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM) under the short-induction conditions. The effects of various IPTG concentrations on DCM (g/L), PHB (g/L), PHB content (% w/w) and PHB productivity (g PHB/(L⋅h)), comparing E. coli JM109 (pColdI-phaCAB$_{A-04}$) and E. coli JM109 (pColdTF-phaCAB$_{A-04}$), are summarized in Table 2. It can be concluded that the optimal concentration of IPTG was 0.5 mM in both cases. The PHB content (% w/w) increased in accordance with the IPTG concentration, but the amount of PHB (g/L) produced was maximum under induction with 0.5 mM IPTG. The PHB content (% w/w) increased approximately 8-fold, and the productivity (g PHB/(L⋅h)) increased 16-fold, compared with those under the control condition in the case of pColdI-phaCAB$_{A-04}$.

### Table 2

Effect of IPTG concentration on DCM (g/L), PHB (g/L), % (w/w) PHB content and PHB productivity in a comparison between E. coli JM109 harboring pColdI-phaCAB$_{A-04}$ and E. coli JM109 harboring pColdTF-phaCAB$_{A-04}$

| Plasmid          | Inoculum % (v/v) | IPTG (mM) | DCM (g/L) | RCM (g/L) | PHB (g/L) | PHB content (% w/w) | Productivity g/(L⋅h) |
|------------------|------------------|-----------|-----------|-----------|-----------|---------------------|----------------------|
| pColdI-phaCAB$_{A-04}$ | short induction   | 5         | 0         | 2.8 ± 0.1 | 2.5 ± 0.1 | 0.3 ± 0.0           | 10.7 ± 1.1           | 0.01 ± 0.00          |
|                  |                  |           | 0.01      | 2.8 ± 0.2 | 1.0 ± 0.2 | 1.8 ± 0.1           | 64.3 ± 3.1           | 0.07 ± 0.03          |
|                  |                  |           | 0.05      | 2.6 ± 0.3 | 0.7 ± 0.2 | 1.9 ± 0.2           | 73.1 ± 3.5           | 0.08 ± 0.04          |
|                  |                  |           | 0.1       | 2.6 ± 0.2 | 0.5 ± 0.1 | 2.1 ± 0.2           | 80.8 ± 0.7           | 0.08 ± 0.05          |
|                  |                  |           | 0.5       | 4.5 ± 0.3 | 0.6 ± 0.1 | 3.9 ± 0.1           | 86.7 ± 2.6           | 0.16 ± 0.07          |
|                  |                  |           | 1         | 2.6 ± 0.1 | 0.4 ± 0.0 | 2.2 ± 0.1           | 84.6 ± 0.6           | 0.09 ± 0.02          |
| continuous induction | 5         | 0.5       | 1.3 ± 0.1 | 0.7 ± 0.0 | 0.6 ± 0.1 | 46.6 ± 3.8           | 0.03 ± 0.01          |
| preinduction     | 5                 | 0.5       | 2.8 ± 0.6 | 0.9 ± 0.3 | 1.9 ± 0.6 | 66.6 ± 4.8           | 0.04 ± 0.01          |
Development of a preinduction method and effect of inoculum size on PHB productivity

We also investigated a preinduction strategy to enhance PHB productivity by extending the PHB production phase at 37 °C for an additional 24 h after conventional induction. When the OD$_{600}$ reached 0.5, IPTG was added at 0.5 mM into the culture, and the temperature was reduced from 37 °C to 15 °C. Then, cultivation was performed for 24 h to allow full expression of the phaCAB$_{A−04}$ protein. Concurrently, the effect of inoculum size (1, 5 and 10% (v/v)) of induced cells was investigated under the preinduction conditions. The results are shown in comparison with those of the conventional induction and short-induction methods (Table 3). The preinduction method with a 5% (v/v) inoculum gave a higher amount of PHB (1.9 ± 0.6 g/L) than conventional induction with an inoculum size of 5% (v/v) (0.6 ± 0.1 g/L) and could extend the productivity of 0.039 ± 0.01 g PHB/(L⋅h) for 48 h so that the PHB content increased from 46.2 ± 3.8% (w/w) to 67.9 ± 4.8% (w/w). The increase in PHB content and PHB productivity occurred with an increase in the inoculum size. Nevertheless, the short-induction method with an inoculum size of 5% (v/v) gave the highest levels of PHB content and productivity. Therefore, the short-induction method using *E. coli* JM109 (pColdI-phaCAB$_{A−04}$) with an inoculum size of 0.5% (v/v) and cultivated until the OD$_{600}$ reached 0.5 (2 h) before induction with 0.5 mM IPTG was selected to investigate the effect of induction temperature in the subsequent experiment.

### Table 3

Comparison of the kinetics of cell growth, (g PHB/g-glucose), and PHB production g/(L⋅h) by *C. necator* strain A-04, *E. coli* JM109 (pColdI-phaCAB$_{A−04}$), *E. coli* JM109 (pColdTF-phaCAB$_{A−04}$), *E. coli* JM109 (pGEX-6P-1-phaCAB$_{A−04}$), *E. coli* JM109 (pBAD/Thio-TOPO-phaCAB$_{A−04}$), and *E. coli* JM109 (pUC19-nativeP-phaCAB$_{A−04}$) in shake flask cultivation.

| Kinetic parameters | pColdI-phaCAB$_{A−04}$ | pColdI-phaCAB$_{A−04}$ | pColdTF-phaCAB$_{A−04}$ | pColdTF-phaCAB$_{A−04}$ | pGEX-6P-1-phaCAB$_{A−04}$ | pBAD/Thio-TOPO-phaCAB$_{A−04}$ | pUC19-nativeP-phaCAB$_{A−04}$ |
|-------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| Temperature (°C)  | 15*                    | 37                     | 15*                    | 37                     | 37                     | 37                     | 37                     |

Short induction was performed with 0.5 mM IPTG at 15 °C for 30 min, and cultivation was performed at 37 °C for 24 h.

Continuous induction was performed with 0.5 mM IPTG at 15 °C, and cultivation was performed at 15 °C for 24 h.

Preinduction was performed with 0.5 mM IPTG at 15 °C for 24 h, and cultivation was performed at 37 °C for 24 h.
|                              | 1.4 ± 0.2 | 2.6 ± 0.2 | 1.3 ± 0.1 | 2.5 ± 0.1 | 0.9 ± 0.2 | 0.8 ± 0.2 | 0.7 ± 0.1 |
|------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Maximum PHB concentration   |           |           |           |           |           |           |           |
| (g/L)                       |           |           |           |           |           |           |           |
| Maximum cell dry weight     | 1.7 ± 0.1 | 2.9 ± 0.2 | 1.7 ± 0.2 | 2.8 ± 0.1 | 1.3 ± 0.1 | 1.2 ± 0.2 | 1.1 ± 0.2 |
| (g/L)                       |           |           |           |           |           |           |           |
| Maximum PHB content         | 85.2 ± 2.5| 89.9 ± 0.8| 84.8 ± 3.3| 90.6 ± 4.3| 69.2 ± 2.6| 66.7 ± 1.8| 63.6 ± 2.2|
| (%wt)                       |           |           |           |           |           |           |           |
| Specific growth rate        | 0.001     | 0.001     | 0.001     | 0.001     | 0.003     | 0.003     | 0.004     |
| (1/h)                       |           |           |           |           |           |           |           |
| Specific consumption rate   | 1.03      | 0.75      | 0.73      | 1.13      | 0.56      | 0.31      | 0.5       |
| (g glucose/g DCM/h)         |           |           |           |           |           |           |           |
| Specific production rate    | 0.09      | 0.19      | 0.07      | 0.29      | 0.05      | 0.03      | 0.05      |
| (g PHB/g DCM/h)             |           |           |           |           |           |           |           |
| (g DCM/g glucose)           | 0.001     | 0.002     | 0.01      | 0.001     | 0.008     | 0.026     | 0.01      |
| (g PHB/g glucose)           | 0.07      | 0.18      | 0.08      | 0.18      | 0.08      | 0.10      | 0.06      |
| Productivity                | 0.03      | 0.09      | 0.03      | 0.10      | 0.02      | 0.03      | 0.02      |
| (g/(L-h))                   |           |           |           |           |           |           |           |
| Time (h)                    | 48        | 30        | 48        | 24        | 48        | 30        | 30        |

The induction was performed with 0.5 mM IPTG at 15 °C for 30 min, and then, cultivation was performed at 37 °C for 48 h.

The induction was performed with 0.5 mM IPTG at 15 °C, and cultivation was performed at 15 °C for 48 h.

Effect of induction temperature on PHB productivity

The optimal short-induction temperature was investigated in a range between 15 °C and 37 °C for 30 min before increasing the temperature to 37 °C for 24 h to confirm that the high PHB productivity resulting in this study is a result of the cold-shock cspA promoter and that 15 °C is the optimal induction temperature. Figure 4 shows the results of the effect of the short-induction temperature (15, 25, 30 and 37 °C) on cell growth and PHB production. It was clear that 15 °C was the optimal induction temperature for enhancing the amount of PHB produced, which resulted in a maximum PHB content of 86.2 ± 2.6% (w/w). The amount of PHB produced decreased as the induction temperature increased, with a concomitant increase in RCM. The cold-shock
temperature promoted PHB production and suppressed RCM. The PHB productivity at 15 °C was 7-fold higher than that obtained with an induction temperature of 37 °C.

**Effect of the TF chaperone on phaCAB**

In phaCAB overexpressing *E. coli* JM109 (pColdI-phaCAB) under the conventional conditions, the formation of inclusion bodies of PhaC~A−04~ has been observed due to the low aqueous solubility of the protein, as described previously [29]. To verify that the cold-shock cspA promoter works together with the TF chaperone to improve the solubility of PhaC~A−04~, the hydrophilic GST tag was fused to the N-terminus of PhaC~A−04~ (pGEX-6P-1-phaCAB~A−04~), and the effect of the GST tag at 37°C on the polymerization reaction of phaC~A−04~ based on the amount of PHB production was investigated. In addition, pBAD/Thio-TOPO-phaCAB~A−04~, encoding a hydrophilic N-terminal thioredoxin fusion protein and C-terminal 6His-fusion protein induced by arabinose, was also used for comparison. The control strain, harboring pUC19-nativeP-phaCAB~A−04~, showed constitutive expression using the native promoter from *C. necator* strain A-04, and no induction agent was required under the same conditions. The phaC~A−04~ protein is expressed *in vivo* and always as a mixture of inclusion bodies and soluble protein; hence, we did not purify the phaC~A−04~ protein and assay its *in vitro* polymerization activity but considered the amount of PHB produced together with the molecular weight of PHB as a result of *in vivo* polymerization activity. The results are shown in Fig. 5 and Table 3. It was clearly found that pColdI-phaCAB~A−04~ and pColdTF-phaCAB~A−04~ gave significantly higher amounts of PHB under the short-induction conditions than under the conventional induction conditions (pGEX-6P-1-phaCAB~A−04~ and pBAD/Thio-TOPO-phaCAB~A−04~). PhaC~A−04~ and TF-PhaC~A−04~ were overexpressed in *E. coli* JM109 cells at both 15°C (conventional method) and 37°C (short induction method). However, the total PhaC~A−04~ protein from pColdI-phaCAB~A−04~ was much higher than that from pColdTF-phaCAB~A−04~ (Fig. 5B). The ratio between the soluble form and inclusion bodies of pColdI-phaCAB~A−04~ was much lower than that of pColdTF-phaCAB~A−04~. Based on our observations, the cold-shock cspA promoter and TF gave functional phaCA-04 protein (Fig. 5C), resulting in the highest specific production, Y_{PS}, and PHB productivity. The PHB production from pGEX-6P-1-phaCAB~A−04~, pBAD/Thio-TOPO-phaCAB~A−04~ and pUC19-nativeP-phaCAB~A−04~ was not different, which may be attributed to the host strain and induction method used in this study.

**Comparison of PHB production between pColdI-phaCAB**

Altogether, for flask cultivation, the optimal conditions were the short-induction method using an inoculum of 0.5% (v/v) in a culture with an OD_{600} of 0.5, cold shock induced with 15 °C for a short time, 30 min, and the addition of 0.5 mM IPTG. These conditions were selected as optimal parameters for scaling up production in a 5-L fermenter. The comparison between pColdI-phaCAB~A−04~ and pColdTF-phaCAB~A−04~ in a 5-L fermenter by the short-induction method was performed because the ratio of the soluble fraction and inclusion bodies of the phaC~A−04~ protein may affect PHB productivity and molecular weight distribution as reported by Harada et al. [29].

Figure 6 shows the time courses of DCM (g/L), RCM (g/L), PHB (g/L), PHB content (% w/w), glucose (g/L), dissolved oxygen (%) and pH during batch cultivation in a 5-L fermenter, comparing *E. coli* JM109 (pColdI-phaCAB~A−04~) (Fig. 6A) and *E. coli* JM109 (pColdTF-phaCAB~A−04~) (Fig. 6B). The soluble PhaC~A−04~ protein detected by western blot analysis was also monitored at 6-h intervals over 48 h. The results shown in Table 4 demonstrated that *E. coli* JM109 (pColdTF-phaCAB~A−04~) was a more effective PHB producer than the other strain. A PHB content of 92.5±5.9% (w/w), PHB production of 8.5±0.8 g/L, DCM production of 9.2±0.3 g/L, Y_{PS} value of 0.40 g PHB/g glucose and productivity of 0.39 g PHB/(L-h) were the maximum values obtained using pColdTF-phaCAB~A−04~, whereas a PHB content of 78.0±2.1% (w/w), PHB production of 5.8±0.1 g/L, DCM production of 7.2±0.3 g/L, Y_{PS} value of 0.32 g PHB/g glucose and productivity of 0.16 g PHB/(L-h) were attained using pColdI-phaCAB~A−04~. The phaC~A−04~ protein produced by pColdTF-phaCAB~A−04~ was more stable and longer lasting (Fig. 6B) than that obtained from pColdI-phaCAB~A−04~, which was no longer detectable after 30 h of cultivation
Therefore, we report here that the short-induction strategy facilitates cold shock cspA and chaperone TF proteins to act synergistically to improve the stabilization of PhaC\textsubscript{A−04} and enhance productivity by 143.8% and the \( Y_{P/S} \) value by 25% in 30 h.

Table 4
Comparison of kinetic parameters, molecular weight, and thermal and mechanical properties of PHB produced by \textit{C. necator} strain A-04, \textit{E. coli} JM109 (pColdI-\textit{phaCAB}\textsubscript{A−04}), \textit{E. coli} JM109 (pColdTF-\textit{phaCAB}\textsubscript{A−04}) and \textit{E. coli} JM109 (pUC19-nativeP-\textit{phaCAB}\textsubscript{A−04})

| Kinetic parameters and polymer properties of PHB | \textit{C. necator} A-04 | pColdTF-\textit{phaCAB}\textsubscript{A−04} | pColdI-\textit{phaCAB}\textsubscript{A−04} | pUC19-nativeP-\textit{phaCAB}\textsubscript{A−04} |
|-----------------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Carbon source (g/L)                           | 20                       | 30                       | 20                       | 20                       |
| Maximum PHB concentration (g/L)               | 5.8 ± 0.5                | 4.7 ± 0.2                | 7.9 ± 0.7                | 5.8 ± 0.1                |
| Maximum cell dry weight (g/L)                 | 7.4 ± 1.5                | 7.3 ± 1.2                | 8.8 ± 0.5                | 7.2 ± 0.3                |
| Maximum PHB content (%wt)                     | 79.0 ± 1.9               | 68.9 ± 2.8               | 90.0 ± 2.3               | 78.0 ± 2.1               |
| Specific growth rate (1/h)                    | 0.003                    | 0.001                    | 0.07                     | 0.06                     |
| Specific consumption rate (g carbon source/g DCM/h) | 0.14                 | 0.05                     | 0.52                     | 0.35                     |
| Specific production rate (g PHB/g DCM/h)      | 0.012                    | 0.019                    | 0.20                     | 0.11                     |
| (g DCM/g carbon source)                       | 0.08                     | 0.03                     | 0.07                     | 0.10                     |
| (g PHB/g carbon source)                       | 0.29                     | 0.35                     | 0.38                     | 0.32                     |
Comparison of the molecular weight and thermal and mechanical properties of PHB produced by pCold-phaCAB<sub>A−04</sub> and pColdTF-phaCAB<sub>A−04</sub> from glucose and crude glycerol

The PHB thin films were subjected to thermal analysis by DSC, molecular weight determination by GPC and mechanical property analysis by a universal testing machine as per the ASTM: D882-91 protocol (Table 4). The PHB from \textit{E. coli} JM109 (pCold-phaCAB<sub>A−04</sub>) had an \(M_W\) of \(8.17 \times 10^5\) Da, an \(M_N\) of \(1.97 \times 10^5\) Da and a PDI of 4.1, whereas the PHB from \textit{E. coli} JM109 (pColdTF-phaCAB<sub>A−04</sub>) had an \(M_W\) of \(2.6 \times 10^5\) Da, an \(M_N\) of \(0.95 \times 10^5\) Da and a PDI 2.8, when glucose was used as a carbon source. However, the PHB from \textit{E. coli} JM109 (pCold-phaCAB<sub>A−04</sub>) obtained using crude glycerol had the lowest \(M_W\) of \(2.42 \times 10^5\) Da, an \(M_N\) of \(0.89 \times 10^5\) Da and a PDI of 2.92. Interestingly, the PHB from \textit{E. coli} JM109 (pUC19-nativeP-phaCAB<sub>A−04</sub>) obtained using crude glycerol showed the highest \(M_W\) of \(9.05 \times 10^5\) Da, an \(M_N\) of \(2.17 \times 10^5\) Da and a PDI of 3.72. The melting temperature, \(T_M\), of all the PHB film samples produced in this study was in the range of 165−178°C [34], and the glass transition temperature, \(T_G\), was in the normal range of 1−4°C [31, 35, 36]. The Young's modulus and tensile strength of the PHB from \textit{E. coli} JM109 (pCold-phaCAB<sub>A−04</sub>) possessed the highest values of 5465 MPa and 56.2 MPa, respectively. Figure 6 shows the morphology and transparency of PHB films produced by \textit{C. necator} strain A-04, \textit{E. coli} JM109 (pCold-phaCAB<sub>A−04</sub>) and \textit{E. coli} JM109 (pColdTF-phaCAB<sub>A−04</sub>). Interestingly, the PHB films prepared by the film casting technique and produced from \textit{E. coli} JM109 (pColdTF-phaCAB<sub>A−04</sub>) showed a soft morphology with high transparency, which was different from the properties of the other PHB films. The PHB films were also subjected to \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR analyses and showed only chemical shifts of the PHB structure.
Discussion

Since 1988, it has been reported that PhaCRe, as a type I synthase and the most intensively studied of these proteins, preferentially catalyzes the polymerization of short-chain (R)-hydroxyalkanoic acids (4 to 6 carbon atoms), particularly the conversion of (R)-3-hydroxybutyrate-coenzyme A (3HBCoA) to poly(hydroxybutyrate) (PHB) [20, 25]. In fact, a high yield of PHB (157 g/L) has been achieved from glucose in high-cell-density cultures of recombinant *E. coli* harboring *phaCABRe* and the additional cell division protein *ftsZ* gene [37, 38]. Ultrahigh-molecular-weight PHB and its applications have also been reported by many research groups [15, 22, 39, 40]. PhaCRe has been used to prepare chimeric enzymes to increase its ability to incorporate MCL monomers for SCL-co-MCL production [41].

Beyond these previous reports, there have been few reports on the application of cold-shock systems for PHB production to address the challenges of soluble and functional phaC expression in *E. coli*. One of the attempts was the use of pCold and a GST-fusion tag to obtain pCold-PhaCRe and pCold-GST-PhaCRe, which were overexpressed in *E. coli* DH5α and *E. coli* BL21(DE3) to investigate the effect of GST fusion on the in vivo solubility of PhaCRe, but the pGEM-T derivative carrying the *pha*Re promoter, His-fused *phaCRe*, *phaARe*, and *phaBRe* and pGEM-GSTphaCReAB were used to evaluate in vitro PHB production [29]. It was revealed that most of the PhaCRe and GST-PhaCRe when overexpressed in *E. coli* BL21(DE3) were detected in the insoluble fraction rather than the soluble fraction, indicating that the solubility of PhaCRe is not improved by GST tag fusion under a cold-inducible promoter. Another recommendation was that a fusion protein to assist in phaC solubilization as well as the 6His-tag should be fused at the N-terminus of phaC because an N-terminal tag has a weaker effect than a C-terminal tag on the polymerization activity of phaC [23, 29, 42].

Although *C. necator* strain A-04 exhibits 99.78% similarity of 16S rRNA, 99.9% similarity of *phaCA*-04 and 100% similarity of *phaA*-04 and *phaB*-04 with those of *C. necator* H16, we observed differences in PHB productivity as well as the monomeric composition of the copolymers and terpolymers when we used the same carbon source [30–32]. Interestingly, *C. necator* strain A-04 also exhibited different growth abilities on pure glycerol as well as crude glycerol compared with *C. necator* strain H16 (data not shown, presented at an international conference). Thus, we initially aimed to use the pColdI and pColdTF expression systems to address the challenges of soluble and functional phaC expression in *E. coli* JM109 and finally evaluate its ability to use crude glycerol as a carbon source for PHB production in a 5-L fermenter. We also investigated the optimal expression conditions and finally compared them with those for other promoters, including the pGEX-6P-1 derivative, carrying N-terminal GST and 6His-fused *phaCAB*-04; the pBAD/Thio-TOPO derivative, carrying C-terminal 6His- and N-terminal thioredoxin-fused *phaCAB*-04; and the constitutive expression vector pUC19, using a native promoter from *C. necator* strain A-04, in both flask cultivation and the 5-L fermenter. The produced PHB was extracted, purified, and prepared using the glass casting method, and subjected to molecular weight analysis and thermal and mechanical property determination to determine the effect of the cold-shock cspA promoter and TF on the functional expression of phaC-04 and PHB products.

First, this study aimed to solubilize PhaC-04 by using the cold-shock cspA promoter and TF to achieve a high yield of soluble recombinant PhaC-04 from *E. coli* JM109. His-tagged phaC-04 was overexpressed by pColdI, but most of the protein was present in insoluble form, with significant aggregation resulting in smear bands (Fig. 2A and 5B), whereas the His-tagged phaC-04-TF fusion protein was expressed from pColdTF at lower levels than the protein from pColdI, but most of this protein was present in soluble form (Fig. 2B and 5B). The ratio of the soluble fraction to the total phaC-04 proteins from pColdTF-phaCAB-04 was much higher than that from pColdI-phaCAB-04. Thus, it can be concluded that the TF chaperone helped solubilize phaC-04 in our investigation, but PHB production and *Yp* in both systems, namely, pColdI-phaCAB-04 and pColdTF-phaCAB-04, were not significantly different. To produce PHB using microbial cells under in vitro conditions, phaC will always naturally be expressed in two forms, namely, insoluble and soluble, and these proteins simultaneously work together under in vitro conditions to perform dynamic PHB polymerization. Thus, in this study, we decided
to consider the amount of PHB, \( Y_{P/S} \), PHB productivity and molecular weight distribution as indirect indicators of PhaC\textsubscript{A−04} activity because there have been many reports that have gained insights into PhaC\textsubscript{Re} expression and PHB production that can be used to support our findings. Focusing on chaperone-assisted PhaC\textsubscript{Re} expression and PHB production, it was previously reported that the TF chaperone (without the cold-shock cspA promoter) was investigated in combination with three chaperone systems for coexpression with PhaC\textsubscript{Re}: GroEL/GroES (plasmid pGro7), TF (plasmid pTF16) and DnaK/DnaJ/GrpE (plasmid pKJE7). Additionally, TF and GroEL/GroES were expressed together (plasmid pG-Tf2), as were GroEL/GroES and DnaK/DnaJ/GrpE (plasmid pG-KJE8) [23]. The study concluded that with the set of strains expressing the N-terminal 6His-tagged fusion protein, the GroEL/GroES system resulted in approximately 6-fold-greater enzyme yields than that obtained in the absence of coexpressed chaperones, whereas TF resulted in approximately 3-fold increases in the soluble protein yield. It seemed likely that although the overexpression of phaC\textsubscript{A−04} was achieved both in terms of quantity (pColdI) and solubility (pColdTF), its function had already reached its limit, so improvement of PHB production was not further observed. Acceleration of PHB productivity would be another goal for reducing the time consumed for microbial cultivation. It was reported that optimization of expression conditions, including inducer concentrations, age of bacterial cells (OD\textsubscript{600}) and induction temperatures, is required to improve PHB productivity.

It was reported that the production level of PhaC was not significantly changed by the addition of IPTG at concentrations greater than 0.1 mM, suggesting that this IPTG concentration is sufficient for pJRDTrc\textsubscript{phaCAB\textsubscript{Re}} to fully express PhaC [28]. In our study, 0.5 mM was the optimal concentration of IPTG for overexpression of phaC\textsubscript{A−04} under the cold-shock cspA promoter, consistent with a previous report [16]. Cells at the initial exponential phase exhibited the highest phaC\textsubscript{A−04} expression level, resulting in enhanced PHB productivity under the short-induction method. We also performed parallel experiments using different hydrophilic tags, including constitutive expression via the native promoter of C. necator strain A-04, N-terminal GST-fused \textit{phaC}A−04, and N-terminal thioredoxin-fused and C-terminal 6His-fused \textit{phaC}A−04, to confirm that the high efficiency of PHB production was contributed by the cold-shock cspA promoter. It was found that N-terminal GST-fused \textit{phaC}A−04, N-terminal thioredoxin-fused and C-terminal 6His-fused \textit{phaC}A−04 and constitutive expression via the native promoter of C. necator strain A-04 gave similar values for DCM production (∼ 1.0 g/L), PHB production (∼0.8 g/L) and PHB content (∼ 65 wt%), which were 2.5 times lower than the values obtained with pCold and pColdTF. Thus, our finding also confirmed that GST-PhaC\textsubscript{A−04} did not improve PHB production and exhibited lower PHB productivity (longer time of polymerization) than the control pUC19-nativeP-\textit{phaC}A−04, as previously mentioned by Harada et al. [29]. The araBAD promoter and N-terminal thioredoxin-fused \textit{phaC}A−04 together with C-terminal 6His-fused \textit{phaC}A−04 showed similar effects on PHB production with pGEX-6P-1-\textit{phaC}A−04 and pUC19-nativeP-\textit{phaC}A−04. However, \textit{E. coli} JM109 (pGEX-6P-1-\textit{phaC}A−04) exhibited higher PHB production than that of pGEM-GST\textit{phaC}ReAB reported previously [29].

It is still unclear why \textit{E. coli} JM109 (pCold-\textit{phaC}A−04) and \textit{E. coli} JM109 (pColdTF-\textit{phaC}A−04) exhibited higher PHB production and productivity than \textit{E. coli} JM109 (pGEX-6P-1-\textit{phaC}A−04), \textit{E. coli} JM109 (pBAD/Thio-TOPO-\textit{phaC}A−04) and \textit{E. coli} JM109 pUC19-nativeP-\textit{phaC}A−04. This may be because the expression of \textit{phaC}A−04 under the cold-shock cspA promoter did not change the hydrophobic interactions between PhaC\textsubscript{A−04} subunits, which are a critical factor involved in dimer formation [29]; however, this needs to be elucidated by determining the quantitative hydrophilicity of the protein surface by using a quartz crystal microbalance (QCM), which is not available in our laboratory. The ratio of soluble to total PhaC\textsubscript{A−04} proteins did not play an important role in PHB productivity, but the TF chaperone resulted in the production of PHB with reduced molecular weights [23] because the M\textsubscript{W} and M\textsubscript{N} of PHB produced by pColdTF were lower than those of pColdI-\textit{phaC}A−04 and pUC19-nativeP- \textit{phaC}A−04.

To achieve low-cost production, crude glycerol as a byproduct from biodiesel production was used as a carbon source to produce PHB using pColdI-\textit{phaC}A−04 and pUC19-nativeP-\textit{phaC}A−04. PHB from \textit{E. coli} JM109 (pColdI-\textit{phaC}A−04) had an M\textsubscript{W} of 2.42 × 10^5 Da and an M\textsubscript{N} of 0.89 × 10^5 Da with a PDI of 2.92. However, the PHB content and PHB productivity were lower than those similarity obtained using glucose. \textit{E. coli} JM109 (pUC19-
nativeP-phaCAB_{A−04}) produced PHB constitutively with a satisfactory $M_W$ of $9.05 \times 10^5$ Da and an $M_N$ of $2.17 \times 10^5$ Da with a PDI 3.72. Typically, PHB obtained from glycerol was reported to have a significantly lower molecular weight than polymers synthesized from other substrates, such as glucose or lactose [43, 44].

A previous study showed that cells grown on glycerol exhibit a highly reduced intracellular state compared to cells grown on glucose under similar dissolved oxygen conditions [45]. Therefore, E. coli JM109 (pColdI-phaCAB\textsubscript{A−04}) and E. coli JM109 (pUC19-nativeP-phaCAB\textsubscript{A−04}) have low DCM production (g/L), PHB production (g/L), PHB content (%wt) and PHB productivity (g/(L-h)) when grown on crude glycerol because glycerol has a significant effect on the intracellular redox state, which causes the cells to direct carbon flow toward the synthesis of highly reduced products to achieve redox balance [46]. It was described in a previous study that the product distributions of E. coli K24K when grown on glucose or glycerol as the substrate were different. The glycerol-grown cultures produced lower amounts of acetate, lactate, and formate and higher amounts of ethanol than those grown on glucose. PHB production from glycerol was lower than that from glucose, except under conditions of low oxygen availability [47]. However, another report revealed that E. coli K24K produced PHBs possessing a $T_G$ of 22°C, whereas the molecular weight of PHB from glycerol was similar to that obtained using glucose [47]. In addition, a study performed using C. necator to produce PHB from commercial glycerol and waste glycerol exhibited products with molecular masses of 957 and 786 kDa, respectively, less than half of the mass of the PHB obtained from glucose [48]. In our study, when pColdI-phaCAB\textsubscript{A−04} expressing E. coli was used to produce PHB from crude glycerol and compared with constitutive pUC19-nativeP-phaCAB\textsubscript{A−04} expressing E. coli, although the amounts of PHB were similar, an $M_W$ of $10.7 \times 10^5$ Da, an $M_N$ of $2.6 \times 10^5$ Da and a PDI of 4.1 were obtained from constitutive pUC19-nativeP-phaCAB\textsubscript{A−04} expressing E. coli, indicating that slow and low expression prolonged and maintained the phaC\textsubscript{A−04} polymerization activity. It was observed that high levels of phaC proteins resulted in high levels of PHB production, but the chain termination reaction of PhaC polymerization activity frequently occurred faster than that observed with retarded and low expression of the phaC protein under the constitutive promoter of pUC19, which in turn resulted in a low amount of PHB with a high molecular weight. In the latter case, the low-level phaC protein slowly utilized the substrate, 3-hydroxybutyric-CoA, via a low-competition reaction with other phaC proteins.

**Conclusion**

This study aimed to improve functional PhaC\textsubscript{A−04} expression levels in E. coli JM109 and found that the cspA promoter in a cold-inducible vector can enhance total PhaC\textsubscript{A−04} expression and TF chaperones showed obvious effects on enhancing PhaC solubility. However, the ratio of soluble to total PhaC\textsubscript{A−04} proteins did not play an important role in PHB productivity, but the TF chaperone resulted in the production of PHB with reduced molecular weights. Thus, the high level of phaC\textsubscript{A−04} resulted in a high amount of PHB, but the chain termination reaction of PhaC polymerization occurred faster than that with the retarded and low expression of phaC\textsubscript{A−04} by the constitutive promoter pUC19, which in turn resulted in a low amount of PHB with a high molecular weight. The findings suggest that PhaC\textsubscript{A−04} is primed by chain elongation and high molecular weight PHB is obtained by adding HB units to the primed PhaC which requires not only PhaC protein but also substantial amount of HB-CoA substrate.

**Materials And Methods**

**Strains and plasmids**

The E. coli strains and plasmids used in this study are listed in Table 1. The PHB-producing C. necator strain A-04 [49] was used to isolate the phaCAB\textsubscript{A−04} gene operon. All bacterial strains were grown at 37 °C in Luria-Bertani (LB) medium supplemented with 100 µg/L ampicillin. The LB medium contained (per liter) 10 g of tryptone (Himedia, Mumbai, India), 5 g of yeast extract (Himedia, Mumbai, India) and 10 g of NaCl (Merck KGaA,
Darmstadt, Germany). Stock cultures were maintained at -80 °C in a 15% glycerol solution. The experiments were performed in a biosafety level 1 laboratory and by researchers and investigators who had undergone biosafety training.

Table 1

| Strains/plasmids        | Relevant description                                                                 | Reference/source                        |
|-------------------------|--------------------------------------------------------------------------------------|----------------------------------------|
| **Strain**              |                                                                                      |                                        |
| *Cupriavidus necator*   | Wild Type                                                                            | [30]                                   |
| strain A-04             |                                                                                      |                                        |
| *Escherichia coli*      | *F′traD36 proA + B + lacI(lacZ)ΔM15/Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17* | Promega Corporation, Madison, WI       |
| JM109                   |                                                                                      |                                        |
| **Plasmid**             |                                                                                      |                                        |
| pUC19                   | Amp′                                                                                  | Thermo Scientific, MA, USA             |
| pColdI                  | Amp′, lacI, cold-shock cspA promoter                                                 | Takara Bio Inc., Shiga, Japan          |
| pColdIF                 | Amp′, lacI, cold-shock cspA promoter and trigger factor                                | Takara Bio Inc., Shiga, Japan          |
| pGEX-6P-1               | Amp′, lacI, tac promoter and glutathione S-transferase (GST)                         | Novagen, WI, USA                       |
| pBAD/Thio-TOPO          | Amp′, araBAD promoter and thioredoxin                                                 | Invitrogen, CA, USA                    |
| pUC19-nativeP-phaCAB_A-04 | pUC19 derivative, carrying phaCAB with native promoter from *C. necator* strain A-04 | This study                             |
| pColdI-phoCAB_A-04      | pColdI derivative, carrying N-terminal 6His-fused phaCAB from *C. necator* strain A-04 | This study                             |
| pColdTF-phoCAB_A-04     | pColdTF derivative, carrying N-terminal 6His-fused phaCAB from *C. necator* strain A-04 | This study                             |
| pGEX-6P-1-phaCAB_A-04   | pGEX-6P-1 derivative, carrying N-terminal GST and 6His-fused phaCAB from *C. necator* strain A-04 | This study                             |
| pBAD/Thio-TOPO-phaCAB_A-04 | pBAD/Thio-TOPO® derivative, carrying C-terminal 6HIS- and N-terminal thioredoxin fused phaCAB from *C. necator* strain A-04 | manuscript in preparation |
| Primer         | Sequence                                                                 | Reference       |
|---------------|---------------------------------------------------------------------------|-----------------|
| pCold-F       | 5′-ATGGATCCCTGAGATGGCGACCGGCAA-3′                                         | This study      |
| pCold-R       | 5′-GTGAATTCAAGCTTTCCAG CCCCATATGCAGG-3′                                    | This study      |
| pGEX-F        | 5′-GGCCCCCTGGGATCCCGGAAAATGGCGACCGGCAA-3′                                 | This study      |
| pGEX-R        | 5′-GCACTCGACTCAGTCAGC CCCCATATGCAGG-3′                                    | This study      |
| nativeP-phaCAB\(\text{A-04}\)-F | 5′-TGGTCCCTGACTG-3′                                                        | This study      |
| nativeP-phaCAB\(\text{A-04}\)-R | 5′-CGTCA CGACCTTGAAT-3′                                                   | This study      |

**Table 5**

Comparison of PHB production by recombinant *E. coli* reported previously

| Strain          | Gene source | Plasmid          | Promoter | Time (h) | Operation | Carbon (g/L) | DCM (g/L) | PHB (g/L) | % PHB (w/w) | Reference |
|-----------------|-------------|------------------|----------|----------|-----------|--------------|-----------|-----------|-------------|-----------|
| JM109           | *C. necator* strain A-04 | pColdI-phaCAB\(\text{A-04}\) | P\(_{\text{cspA}}\) | 36       | Batch     | 2% (w/v) glucose | 7.2 ± 0.3 | 5.8 ± 0.1 | 78.0 ± 2.1 | 0.32      | This study |
| JM109           | *C. necator* strain A-04 | pColdTF-phaCAB\(\text{A-04}\) | P\(_{\text{cspA}}\) | 18       | Batch     | 2% (w/v) glucose | 8.6 ± 0.3 | 7.0 ± 0.3 | 82.9 ± 0.3 | 0.29      | This study |
| JM109           | *C. necator* strain A-04 | pColdTF-phaCAB\(\text{A-04}\) | P\(_{\text{cspA}}\) | 30       | Batch     | 2% (w/v) glucose | 8.8 ± 0.5 | 7.9 ± 0.7 | 90.0 ± 2.3 | 0.38      | This study |
| DH5α            | *C. necator* H16 | pQKZ103          | P\(_{\text{rpoS}}\) | 48       | Flask     | 1.5% (w/v) glucose | 4.1     | 3.52      | 85.8        | -         | [54]       |
| JW2294          | *C. necator* H16 | pWYC09           | P\(_{\text{adhE}}\) | 24       | Batch     | w/o air supply | 7.8 ± 1.8 | 5.0 ± 1.5 | 64.3 ± 7.4 | -         | [55]       |
| BW25113         | *C. necator* H16 | pWYC09           | P\(_{\text{adhE}}\) | 24       | Batch     | w/o air supply | 6.7 ± 1.6 | 3.0 ± 1.3 | 45.5 ± 3.9 | -         | [55]       |
Construction of recombinant plasmids

The phaCAB<sub>A−04</sub> operon PHB biosynthetic genes from C. necator A-04 were PCR-amplified using the following pair of primers: forward primer 5′-ATGGATCCCTCGAGATGGCAACGGCAAG-3′ (the XhoI site is underlined) and reverse primer 5′-GTAAGCTTTCGACCATGCAGGCC-3′ (the HindIII site is underlined). Primers were designed based on accession numbers FJ897463, FJ897461 and FJ897462. The blunted PCR product was purified and subcloned into pBluescript SK− (Stratagene, La Jolla, CA, USA) linearized by SmaI. The recombinant plasmid digested with XhoI and HindIII was cloned into cold-shock-inducible pColdI and pColdTF vectors (Takara Bio Inc., Shiga, Japan) at the Xhol and HindIII restriction sites, yielding pColdI-phaCAB<sub>A−04</sub> and pColdTF-phaCAB<sub>A−04</sub>, respectively. For the plasmid pGEX-6P-1-phaCAB<sub>A−04</sub>, the phaCAB<sub>A−04</sub> operon was amplified by the primers pGEX-F and pGEX-R (Table 1). The 3885-bp DNA fragment was digested by BamHI and XhoI and cloned into BamHI-XhoI-digested pGEX-6P-1 to obtain pGEX-6P-1-phaCAB<sub>A−04</sub>. To construct the constitutive expression vector pUC19-nativeP-phaCAB<sub>A−04</sub>, the primers nativeP-phaCAB<sub>A−04</sub>-F and nativeP-phaCAB<sub>A−04</sub>-R were used to amplify the phaCAB<sub>A−04</sub> operon, including its native promoter. The blunted PCR product was purified and cloned into SmaI-linearized pUC19 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), yielding pUC19-nativeP-phaCAB<sub>A−04</sub>. PCRs were performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). E. coli JM109 was used as a host for cloning and PHB production. The accuracy of the constructed plasmid was verified by the corresponding restriction enzyme and sequencing.

Optimization of culture conditions for PHB production in shake flask cultivation

Expression vectors named pColdI-phaCAB<sub>A−04</sub> and pColdTF-phaCAB<sub>A−04</sub> with the entire phaCAB<sub>A−04</sub> operon were transformed into E. coli JM109 by the heat shock method [50]. Shake flask experiments were performed in 250-mL Erlenmeyer flasks containing 50 mL of medium. E. coli JM109 cells transformed with pColdI-phaCAB<sub>A−04</sub>
or pColdTF-phaCAB<sub>A−04</sub> were grown in LB medium containing ampicillin (100 µg/mL) on a rotary incubator shaker (Innova 4300, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at 37 °C and 200 rpm for 24 h. The overnight seed culture was inoculated into fresh LB medium (5% v/v inoculum) containing 100 µg/L ampicillin and 20 g/L glucose prior to induction with temperature and IPTG, separately using three different induction methods (Fig. 1).

For the synthesis of PHB using the conventional induction method, the procedure was performed according to the user manual (Takara Bio Inc., Otsu, Shiga, Japan). The culture was incubated at 37 °C and 200 rpm until the optical density at 600 nm (OD<sub>600</sub>) reached 0.5, 1.3, 2.1 and 2.4. Next, the cultivation temperature was reduced from 37 °C to 15 °C for 30 min. The expression of the phaCAB operon was induced by the addition of 0.5 mM IPTG, and cultivation was continued at 15 °C for an additional 24 h.

For the synthesis of PHB using the short-induction method developed in this study, the culture was incubated at 37 °C and 200 rpm until the OD<sub>600</sub> reached 0.5, 1.3, 2.1 and 2.4. Then, the temperatures were varied at 15, 25, 30 and 37 °C for 30 min. Next, the expression of the phaCAB operon was induced by adding various concentrations (0.01, 0.05, 0.1, 0.5 and 1.0 mM) of IPTG, and the cultivation was maintained at 37 °C for 24 h.

For the synthesis of PHB using the preinduction method developed in this study, the culture was incubated at 37 °C and 200 rpm until the OD<sub>600</sub> reached 0.5. Then, 0.5 mM IPTG was added to the culture and the temperature was reduced from 37 °C to 15 °C for 24 h. The induced cells were harvested by centrifugation, the medium was discarded, and the cells were resuspended in an equal volume of fresh LB medium. Then, the induced cells at 1, 5 or 10% (v/v) were transferred into fresh LB medium supplemented with 100 µg/L ampicillin and 20 g/L glucose and incubated at 37 °C and 200 rpm for 24 h.

For comparison of the effect of phaC expression on PHB production under various types of promoters, fusion proteins and chaperones, shake flask experiments were performed in 250-mL Erlenmeyer flasks containing 50 mL of LB medium containing ampicillin (100 µg/mL) on a rotary incubator shaker at 37 °C and 200 rpm for 24 h. For PHB production, overnight cultures in LB medium (1 mL) were transferred into fresh LB medium supplemented with glucose (20 g/L) and ampicillin (100 µg/mL). Recombinant <i>E. coli</i> JM109 (pColdi-phaCAB<sub>A−04</sub>) and <i>E. coli</i> JM109 (pColdTF-phaCAB<sub>A−04</sub>) were induced to produce PHB using the conventional induction method and short-induction method. The effect of GST (the hydrophilic fusion protein) and the tac promoter on PHB production was investigated using <i>E. coli</i> JM109 (pGEX-6P-1-phaCAB<sub>A−04</sub>), which was induced by the addition of IPTG (0.5 mM). The effect of the araBAD promoter and N-terminal thioredoxin fusion protein together with the C-terminal 6His-fusion protein on phaC and PHB production was examined by inducing <i>E. coli</i> JM109 (pBAD/ThioTOPO-phaCAB<sub>A−04</sub>) with arabinose (1% w/v). <i>E. coli</i> JM109 (pUC19-nativeP-phaCAB<sub>A−04</sub>), which exhibits constitutive expression, was used as a control strain. All these comparison experiments were performed at 15 °C or 37 °C for 48 h.

**Conditions for PHB production in a 5-L fermenter**

A preculture was prepared in 500-mL Erlenmeyer flasks containing 100 mL of LB medium and grown on a rotary shaker at 37 °C at 200 rpm for 24 h. The preculture was inoculated into a 5-L bioreactor (MDL500, B.E. Marubishi Co., Ltd., Tokyo, Japan) containing 2 L of LB medium supplemented with 100 µg/L ampicillin and 20 g/L glucose at an inoculation volume of 5% (v/v). The agitation speed and the air flow rate were 500 rpm and 1 mL/min, respectively. After an OD<sub>600</sub> of 0.5 was obtained, the cultivation temperature was reduced from 37 °C to 15 °C for 30 min. Next, IPTG was added to the culture at a final concentration of 0.5 mM. After IPTG addition, the cultivation temperature was shifted from 15 °C to 37 °C and maintained at 37 °C for 48 h. Culture samples were collected at 6 h intervals for 48 h.

**Analytical methods**

Cell growth was monitored by the DCM, which was determined by filtering 5 mL of the culture broth through preweighed cellulose nitrate membrane filters (pore size = 0.22 µm; Sartorius, Goettingen, Germany). The filters were dried at 80 °C for 2 days and stored in desiccators. The net biomass was defined as the residual cell mass
(RCM), which was calculated by subtracting the amount of PHB from the DCM. The PHB in dried cells was methyl-esterified using a mixture of chloroform and 3% (v/v) methanol-sulfuric acid (1:1 v/v) [51]. The resulting monomeric methyl esters were quantified by a gas chromatograph (model CP3800, Varian Inc., Walnut Creek, CA, USA) using a Carbowax-PEG capillary column (0.25-μm df, 0.25-mm ID, 60-m length, Varian Inc.). The internal standard was benzoic acid, and the external standard was PHB (Sigma-Aldrich Corp.). The total reducing sugar concentration was determined using a 3,5-dinitrosalicylic acid (DNS) assay [52].

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis.**

Recombinant *E. coli* cells were cultured with and without induction. Cells were collected by centrifugation at 17,000 × g and 4 °C for 30 min. Cell pellets were resuspended in 100 mM Tris-HCl (pH 8.0) and normalized to an OD<sub>600</sub> of 2.0. Total proteins were extracted from cells by using a sonicator (Sonics Vibra Cell VCX 130, Sonics & Materials, Inc., Newtown, CT, USA). The internal standard was benzoic acid, and the external standard was PHB (Sigma-Aldrich Corp.). The total reducing sugar concentration was determined using a 3,5-dinitrosalicylic acid (DNS) assay [52].

**Analysis of polymer molecular weight**

The molecular weight was determined by gel permeation chromatography (GPC; Shimadzu 10A GPC system, Shimadzu Co., Ltd., Kyoto, Japan) with a 10A refractive index detector and two Shodex columns (GPC K-806M columns; 8.0 mm ID × 300 mm L, Showa Denko K.K., Tokyo, Japan). The polymer was dissolved in 0.1% (w/v) chloroform and filtered through a 0.45-μm Durapore® (PVDF) membrane filter with low protein binding capacity (Millex®-HV, Merck Millipore Ltd., Tullagreen, Carrigtwohill Co., Cork, Ireland). The temperature was 40°C, and the flow rate was 0.8 mL/min. A standard curve was determined for polystyrene with low polydispersity under the same conditions for molecular weights of 1.26 × 10<sup>3</sup>, 3.39 × 10<sup>3</sup>, 1.30 × 10<sup>4</sup>, 5.22 × 10<sup>4</sup>, 2.19 × 10<sup>5</sup>, 7.29 × 10<sup>5</sup>, 2.33 × 10<sup>6</sup> and 7.45 × 10<sup>6</sup>. The M<sub>W</sub> and the number-average molecular weight (M<sub>N</sub>) were determined by GPC, and the polydispersity index (PDI) was calculated as the ratio.

**Preparation of PHB films**

PHB films were prepared according to the ASTM: D882-91 protocol. The PHB films were prepared from chloroform solutions of the polyesters using conventional solvent-casting techniques and a glass tray (Pyrex, Corning Incorporated, Corning NY, USA) as the casting surface (modified from [53]). The thickness of the thin polyester films was regulated by controlling the concentration of the polymer in chloroform (1% w/v) and the volume of the polymer solution. The thickness of the PHB films was 0.05 mm, which was confirmed using a caliper (Model 500 – 175: CD-12C, Mitutoyo Corporation, Kawasaki-shi, Kanagawa, Japan). Film samples were aged for 1 month in a desiccator at ambient temperature to allow them to reach crystallization equilibrium.

**Analysis of the mechanical properties of PHB films**

The mechanical tests were conducted at the Scientific and Technological Research Equipment Center, Chulalongkorn University, using a universal testing machine (H10KM, Wuhan Huatian Electric Power Automation Co., Ltd., Wuhan, China) with a crosshead speed of 10 mm/min. The variables measured included the elongation at the break point (%), the stress at maximal load (MPa), and the Young’s modulus (MPa). The data represent the mean values for ten samples tested under the same conditions.
Thermal analysis by differential scanning calorimetry (DSC)

A 10-mg sample of PHB was encapsulated in an aluminum sample vessel and placed in the sample holding chamber of the DSC apparatus (DSC7, PerkinElmer, Inc., Waltham, MA, USA). STARE® software (version SW 10.00; Mettler-Toledo International Inc., Columbus, OH, USA) was used to operate the DSC apparatus at the Petroleum and Petrochemical College, Chulalongkorn University. The previous thermal history of the sample was removed before the thermal analysis by heating the sample from ambient temperature to 180°C at 10°C/min. Next, the sample was maintained at 180°C for 5 min before cooling at 10°C/min to −50°C. The sample was then thermally cycled at 10°C/min to 180°C. The melting peak temperature, denoted by \( T_M \), was given by the intersection of the tangent to the furthest point of an endothermic peak and the extrapolated sample baseline. The glass transition temperature, denoted by \( T_G \), could be estimated by extrapolating the midpoint of the heat capacity difference between glassy and viscous states after heating of the quenched sample.

Data analysis

All the data presented in this manuscript are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). One-way analysis of variance (ANOVA) followed by Duncan’s test for testing differences among means was conducted using SPSS version 22 (IBM Corp., Armonk, NY, USA). Differences were considered significant at \( P < 0.05 \).

Future Research Directions

Recently, state-of-the-art technology has been applied in the development of recombinant technologies for PHB production to replace fossil-derived plastics with competitive green technologies for PHB production. Projects are ongoing in our laboratory to develop a technoeconomic platform for PHB production in both wild-type and recombinant strains from sustainable feedstocks such as glycerol waste from the biodiesel industry. Optimization based on high-cell-density cultivation will be conducted in fed-batch cultivation to enhance productivity and shorten cultivation time. The green extraction and purification process developed in previous work will be integrated into this framework. Purified PHB is used in the development of innovative technologies for applications in microbeads and packaging.

Abbreviations

NA: Not Applicable; PHAs: polyhydroxyalkanoates; PHB: polyhydroxybutyrate; DCW: dry cell mass; RCM: residual cell mass; DSC: differential scanning calorimetry; GPC: gel permeation chromatography; \( \eta \): yield coefficient of PHB produced from consumed PHB substrate (g PHB/g PHB substrate); \( \eta \): yield coefficient of the residual cell mass produced from the consumed PHB substrate (g RCM/g PHB substrate)

Declarations

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Authors’ contributions

TB performed the experiments and discussed the results. RWS provided guidance for the experimental design and discussed the results. KH provided suggestions for the experimental design and discussed the results. SCN provided guidance and suggestions for the experimental design, discussed the results, and wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials

PHA biosynthesis operon of C. necator strain A-04 consisted of three genes sequences were uploaded to GenBank® (acetyl-CoA acetyltransferase (phaA<sub>A−04</sub>, 1182 bp, 40.6 kDa, accession no. FJ897461), acetoacetyl-CoA reductase (phaB<sub>A−04</sub>, 741 bp, 26.4 kDa, accession no. FJ897462) and PHB synthase (phaC<sub>A−04</sub>, 1770 bp, 64.3 kDa, accession no. FJ897463).

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors certify that no actual or potential conflicts of interest in relation to this article exist.

Consent for publication

The authors agree to publish in the journal.

Conflict of interest

The authors certify that no actual or potential conflict of interest exists in relation to this article.

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Figure 1. Bacterial strain E. coli JM109 harbouring pColdI-phaCAB_{A-04} and pColdTF-phaCAB_{A-04} was grown in LB +100 µg/L ampicillin at 37°C.

\[ \text{OD}_{600} 0.5, 1.3, 2.1, 2.4 \]

\[ \text{LB} + 2\% \text{glucose} + 100 \mu g/L \text{ ampicillin, } 15^\circ \text{C, for 30} \]

0.01, 0.05, 0.1

0.5 mM IPTG

harvest induced

\[ \text{LB} + 2\% \text{glucose} + 100 \mu g/L \text{ ampicillin, } 15^\circ \text{C, for } 30 \]

\[ \text{OD}_{600} 0.5 \]

\[ \text{LB} + 2\% \text{glucose} + 100 \mu g/L \text{ ampicillin, } 15^\circ \text{C, for } 30 \]

\[ \text{cspA} \]

\[ \text{phaCAB} \]

phaC, phaA, phaB proteins are induced with 15°C.
Schematic of three different induction methods for heterologous expression of the phaCABA-04 biosynthesis operon in E. coli JM109 (pColdI-phaCABA-04) and E. coli JM109 (pColdTF-phaCABA-04). (A) Conventional induction method: the culture was incubated at 37°C and 200 rpm until the OD600 reached 0.5, 1.3, 2.1 and 2.4. Then, the cultivation temperature was decreased from 37°C to 15°C for 30 min, and the expression of the phaCABA-04 operon was induced by the addition of 0.5 mM IPTG. The cultivation temperature was further maintained at 15°C for 24 h. (B) Short-induction method: the culture was incubated at 37°C and 200 rpm until the OD600 reached 0.5, 1.3, 2.1 and 2.4. Then, the temperatures were varied at 15, 25, 30 and 37°C for 30 min. Next, the expression of the phaCABA-04 operon was induced by adding various concentrations (0.01, 0.05, 0.1, 0.5 and 1.0 mM) of IPTG, and the cultivation temperature was maintained at 37°C for 24 h. (C) Preinduction method: the culture was incubated at 37°C and 200 rpm until the OD600 reached 0.5. Next, 0.5 mM IPTG was added into the culture when the temperature was decreased from 37°C to 15°C for 24 h. Then, the induced cells were harvested by centrifugation, the medium was discarded, and the cells were resuspended in an equal volume of fresh LB medium. Finally, the induced cells at 1, 5 or 10% (v/v) were transferred into fresh LB medium supplemented with 100 g/L ampicillin and 20 g/L glucose and incubated at 37°C and 200 rpm for 24 h.
Figure 2.1

Figure 2
Effect of the growth phase suitable for cold-shock induction on DCM and PHB content (% w/w) under the continuous-induction method. The different growth phases were investigated by varying OD600 based on cultivation time (0.5 (2 h, early exponential phase), 1.3 (4 h, middle exponential phase), 2.1 (6 h, late exponential phase) and 2.4 (10 h, stationary phase)) for (A) E. coli JM109 (pColdI-phaCABA-04) and (B) E. coli JM109 (pColdTF-phaCABA-04). A control experiment was performed with 0.0 mM IPTG induction. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs). The PhaCA-04 protein was detected by western blot analysis using anti-His tag antibody as the primary antibody. The band appearing in the western blot at the position corresponding to that of the His-tagged phaCA-04 protein was 67 kDa in size for pColdI-phaCABA-04, and the fusion protein of His-tagged phaCA-04 and TF was 115 kDa in size. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs).
Figure 3
Effect of the growth phase suitable for cold-shock induction on DCM and PHB content (% w/w) under the short-induction method. The different growth phases were investigated by varying OD600 based on cultivation time (0.5 (2 h, early exponential phase), 1.3 (4 h, middle exponential phase), 2.1 (6 h, late exponential phase) and 2.4 (10 h, stationary phase)) for (A) E. coli JM109 (pColdI-phaCABA-04) and (B) E. coli JM109 (pColdTF-phaCABA-04). A control experiment was performed with 0.0 mM IPTG induction. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs).
Figure 4.
Effect of different short-induction temperatures (15, 25, 30 and 37°C) on the DCM (g/L), RCM (g/L), PHB (g/L) and PHB content (% w/w) of E. coli JM109 (pColdI-phaCABA-04). All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs).

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Figure 5

PHB production by E. coli JM109 (pColdI-phaCABA-04), E. coli JM109 (pColdTF-phaCABA-04), E. coli JM109 (pGEX-6P-1-phaCABA-04), E. coli JM109 (pBAD/Thio-TOPO-phaCABA-04) and E. coli JM109 (pUC19-nativeP-phaCABA-04) in shake flask cultivation. (A) Time courses of PHB production (g/L). (B) The total PhaCA-04 protein was confirmed by SDS-PAGE analysis (20 µg of total protein was loaded in each lane). (C) The soluble PhaCA-04 protein was confirmed by SDS-PAGE analysis (20 µg of total protein was loaded in each lane). Lane M, Protein molecular weight marker; lane 1, E. coli JM109 (pUC19-nativeP-phaCABA-04) at 37°C; lane 2 E. coli JM109 pBAD/Thio-TOPO-phaCABA-04 at 37°C; lane 3 E. coli JM109 (pGEX-6P-1-phaCABA-04) at 37°C; lane 4 E. coli JM109 (pColdI-phaCABA-04) at 37°C; lane 5 E. coli JM109 (pColdTF-phaCABA-04) at 37°C. The band appearing in the SDS-PAGE at the position corresponding to that of the phaCA-04 protein was 64 kDa in size for pUC19-
nativeP-phaCABA-04, His-tagged phaCA-04 fusion protein was 67 kDa in size for pColdI-phaCABA-04, Thioredoxin-tagged phaCA-04 fusion protein was 77 kDa in size for pBAD/Thio-TOPO-phaCABA-04, GST-tagged phaCA-04 fusion protein was 91 kDa in size for pGEX-6P-1-phaCABA-04, and the fusion protein of His-tagged phaCA-04 and TF was 115 kDa in size for pColdTF-phaCABA-04.
Figure 6

Time courses of DCM (g/L), RCM (g/L), PHB (g/L), PHB content (% w/w), and glucose (g/L) and pH during batch cultivation in a 5-L fermenter under the short-induction method in a comparison between (A) E. coli JM109 (pColdI-phAABA-04) and (B) E. coli JM109 (pColdTF-phAABA-04). The band appearing in the western blot at the position corresponding to that of the His-tagged phaCA-04 protein was 67 kDa in size for pColdI-phAABA-04, and the fusion protein of His-tagged phaCA-04 and TF was 115 kDa in size. All the data are representative of the results of three independent experiments and are expressed as the mean values ± standard deviations (SDs).
Morphology of PHB films produced by (A) C. necator strain A-04, (B) E. coli JM109 (pColdI-phaCABA-04) and (C) E. coli JM109 (pColdTF-phaCABA-04).