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BIOENERGY/BIOFUELS/BIOCHEMICALS - ORIGINAL PAPER

Increasing L-threonine production in *Escherichia coli* by overexpressing the gene cluster *phaCAB*

Jianli Wang[1,2] · Wenjian Ma[1,2] · Yu Fang[1,2] · Jun Yang[1,3] · Jie Zhan[1,2] · Shangwei Chen[1] · Xiaoyuan Wang[1,2,3]

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Abstract

L-Threonine is an important branched-chain amino acid and could be applied in feed, drugs, and food. In this study, L-threonine production in an L-threonine-producing *Escherichia coli* strain TWF001 was significantly increased by overexpressing the gene cluster *phaCAB* from *Ralstonia eutropha*. TWF001/pFW01-*phaCAB* could produce 96.4-g/L L-threonine in 3-L fermenter and 133.5-g/L L-threonine in 10-L fermenter, respectively. In addition, TWF001/pFW01-*phaCAB* produced 216% more acetyl-CoA, 43% more malate, and much less acetate than the vector control TWF001/pFW01, and meanwhile, TWF001/pFW01-*phaCAB* produced poly-3-hydroxybutyrate, while TWF001/pFW01 did not. Transcription analysis showed that the key genes in the L-threonine biosynthetic pathway were up-regulated, the genes relevant to the acetate formation were down-regulated, and the gene *acs* encoding the enzyme which converts acetate to acetyl-CoA was up-regulated. The results suggested that overexpression of the gene cluster *phaCAB* in *E. coli* benefits the enhancement of L-threonine production.

Keywords L-Threonine production · *Escherichia coli* · Poly-3-hydroxybutyrate · *phaCAB* · Acetate

Introduction

L-Threonine is an essential branched-chain amino acid, and has been widely used in food, feed, and pharmaceutical industries [7, 26]. L-Threonine production could be raised to a much higher level using *Escherichia coli* than using *Corynebacterium glutamicum* [6, 19, 42]. In *E. coli*, L-threonine biosynthesis involves six steps, starting from oxaloacetate (Fig. 1) [6, 45]. Oxaloacetate can be made from acetyl-CoA through tricarboxylic acid (TCA) cycle, and converted to L-aspartate. L-aspartate can be phosphorylated by aspartate kinases I, II, or III which are encoded by *thrA*, *metL,* or *lysC*, respectively [8]. Aspartate kinases I and II can also catalyze aspartyl semialdehyde to form homoserine. Homoserine can be phosphorylated by homoserine kinase encoded by *thrB*. Then, threonine synthase encoded by *thrC* converts homoserine phosphate into L-threonine. Intracellular L-threonine can be secreted by L-threonine export proteins RhtA, RhtB, or RhtC [15, 42, 43]. The strategy of overexpression of the *thrABC* cluster is frequently used to increase L-threonine production in *E. coli* [6, 16, 17, 28, 32, 45, 46].

Oxaloacetate is the initial metabolite of all L-aspartate family amino acids [6, 45]. It is a component of TCA cycle, which starts from reaction of acetyl-CoA and oxaloacetate [41]. It could be made from pyruvate or phosphoenol pyruvate through the anaplerotic reaction or from malate [2, 41, 46]. However, the metabolic flux towards pyruvate and acetyl-CoA can be easily directed to acetate by enzymes encoded by *poxB* and *pta-ackA*, respectively (Fig. 1) [10, 37]. Therefore, obtaining high amount of oxaloacetate and maintaining low concentration of acetate is important for L-threonine production in *E. coli* [45]. Acetate is one of the major by-products in L-threonine-producing *E. coli*, and its overproduction significantly retards the cell growth. Acetate can be converted into acetyl-CoA by acetyl-CoA synthetase encoded by *acs* (Fig. 1). The overexpression of *acs* could significantly improve L-threonine production [18]. Enhancing the glyoxylate shunt to accumulate

✉ Xiaoyuan Wang
xwang@jiangnan.edu.cn

1 State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China

2 International Joint Laboratory on Food Safety, Jiangnan University, Wuxi 214122, China

3 Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

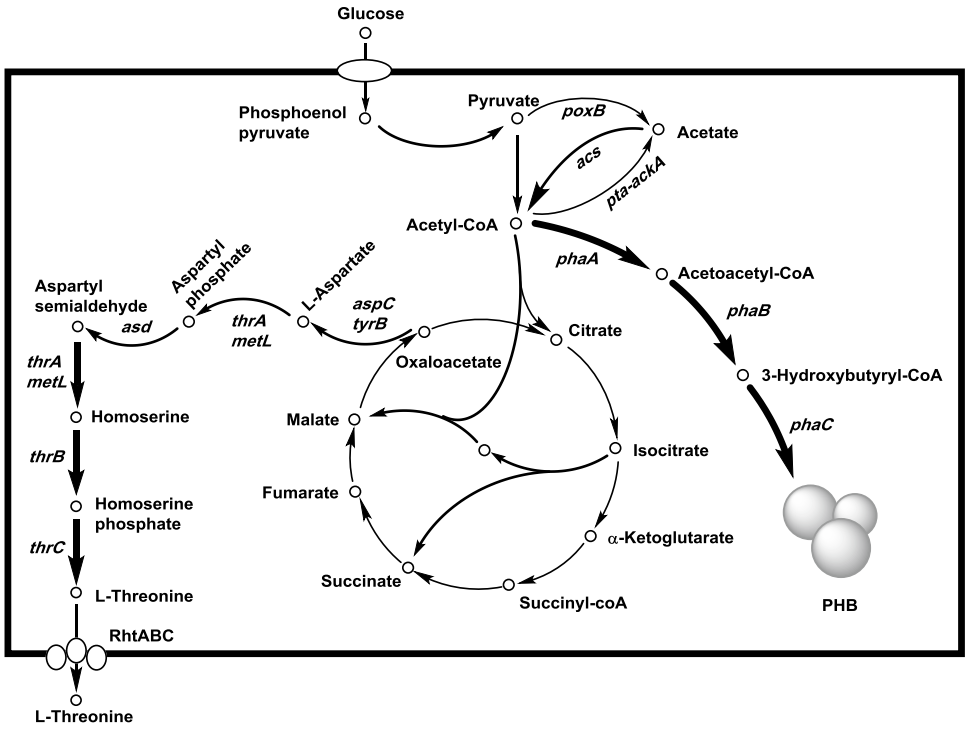

Fig. 1 L-Threonine biosynthetic pathway and introduced PHB biosynthesis pathway in *E. coli*. Genes coding for corresponding metabolic enzymes are shown in italic

oxaloacetate [46], increasing acetyl-CoA supply [18], overcoming acetate overflow [42], and improving the generation of cofactors [23] could also improve L-threonine production [42].

Poly-3-hydroxybutyrate (PHB) is a biopolymer consisting of linear chains of (R)-3-hydroxybutyrate units [20, 30, 47]. It can be biosynthesized from acetyl-CoA by enzymes β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase encoded by *phaA*, *phaB,* and *phaC*, respectively [1, 21]. PHB biosynthesis in *E. coli* could be improved by engineering the L-threonine bypass [22, 37]. PHB accumulation in *E. coli* could reduce the level of acetate and improve the production of succinate [12, 13], L-tryptophan [10], or 5-aminolevulinic acid [44]. Overexpression of the gene cluster *phaCAB* from *Ralstonia eutropha* in *Corynebacterium glutamicum* could increase L-glutamate production by 68% and reduce the accumulation of by-products α-ketoglutarate, glutamine, and lactic acid [24]. Overexpression of *phaCAB* in *Corynebacterium crenatum* could increase L-arginine production 20.6% [40]. Overexpression of *phaCAB* in L-isoleucine producing *C. glutamicum* WM001 produced 44% more L-isoleucine and 30% more acetyl-CoA [29]. Overexpression of *phaCAB* in *E. coli* could produce 11.6% more L-tryptophan and led to the up-regulation of the genes in the L-tryptophan operon [10]. Overexpression of *phaCAB* could also increase succinate in *E. coli* MG1655 [13] and 5-aminolevulinic acid production in *E. coli* DH5α [44]. However, increasing L-threonine production in *E. coli* by overexpression of the gene cluster *phaCAB* has not been reported.

In this study, the influence of overexpression of the gene cluster *phaCAB* on L-threonine production in *E. coli* TWF001 [46] has been investigated. The gene cluster *phaCAB* from pBHR68 [33] was inserted into the triclosan-resistant plasmid pFW01 [46], and introduced into TWF001. L-Threonine production and the acetate accumulation in TWF001/pFW01-*phaCAB* were investigated.

Materials and methods

Bacteria and culture conditions

Bacteria and plasmids used in this work are listed in Table 1. *E. coli* cells were grown at 37 °C in LB medium (5-g/L yeast extract, 10-g/L tryptone, and 10-g/L NaCl) with 200-rpm shaking. Triclosan (1 μM) was added when necessary. L-threonine-producing *E. coli* strain TWF001 (CCTCC no. M2017730) [46] was originally isolated from soil, and is closely related to *E. coli* MG1655, based on their 16S rDNA sequences.

DNA preparation and PCR techniques

Restrictions enzymes, T4 DNA ligase, and DNA Ladder were purchased from Sangon (Shanghai, China). PrimeSTAR™ HS DNA polymerase was purchased from TaKaRa (Dalian, China). Plasmid Minipreps Purification System B used for isolating the plasmid DNA from *E. coli* was from

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Sources
Strains		
JM109	*endA*1, *recA*1, *gyr*A96, *thi*-1, *hsdR*17 (rk−, mk+), *relA*1, *supE*44, Δ(*lac-proAB*)/F′[*traD*36 *proAB laq I*q *lacZ*ΔM15]	NEB
TWF001	L-Threonine-producing *E. coli* strain	[46]
TWF001/pFW01	TWF001 containing pFW01	This study
TWF001/pFW01-*phaCAB*	TWF001 containing pFW01-*phaCAB*	This study
Plasmids		
pBHR68	Derived from pBluescript SK-, harboring the *phaCAB* from *Ralstonia eutropha*, Amp^R	[33]
pFW01	Derived from pBluescript SK-, harboring the *fabV* gene, $Triclosan^R$	[46]
pFW01-*phaCAB*	Derived from pFW01, harboring the *phaCAB* amplified from pBHR68	This study

BioDev-Tech (Beijing, China). TIAnamp Bacteria DNA Kit used for isolating the genomic DNA from *E. coli* was from Tiangen (Beijing, China). EZ-10 Spin Column DNA Gel Extraction Kit used for purifying DNA from agarose gels was purchased from Bio Basic Inc. (Markham, Canada). DNA synthesis and sequencing were performed by Sangon (Shanghai, China).

PCR experiments were performed using Mastercycler from Eppendorf (Hamburg, Germany). The sequences of all primers used in this study are listed in Table 2.

Construction of plasmid pFW01-*phaCAB*

The gene cluster *phaCAB* for poly-3-hydroxybutyrate biosynthesis was cloned using pBHR68 [33] as a template and inserted in pFW01, resulting in the plasmid pFW01-*phaCAB*. Briefly, the *phaCAB* fragment was cloned with primers *phaCAB*-F/*phaCAB*-R, and then digested with *Eco*RI *and Hin*dIII, and pFW01 was digested with *Eco*RI *and Hin*dIII and then ligated together, resulting in the plasmid pFW01-*phaCAB*. The correct sequence in pFW01-*phaCAB* was checked by colony PCR, restriction enzyme digestion, and DNA sequencing.

The plasmids pFW01 and pFW01-*phaCAB* were transformed into *E. coli* strain TWF001, resulting in TWF001/pFW01 and TWF001/pFW01-*phaCAB*, respectively.

Quantification of mRNA using real-time PCR (RT-PCR)

RT-PCR was used to quantify mRNA levels of *aceA*, *aceB*, *aspC*, *thrA*, *thrB*, *thrC*, *poxB*, *pta,* and *ackA* in TWF001/pFW01-*phaCAB* using TWF001/pFW01 as the control. Total RNA was extracted from *E. coli* cells grown at the late-exponential phase using an RNA extraction kit (Bio Flux, Beijing, China). Residual DNA was removed from the RNA sample by DNase I. The quality and amount of RNA were judged and quantified by electrophoresis. Using random hexamer primers, 500-ng RNA was reversely transcribed into cDNA using a Revert Aid™ First Strand cDNA synthesis kit (Fermentas, Shanghai, China). RT-PCR was performed using an ABI Step One RT-PCR system (Applied Biosystems, San Mateo, CA, USA) with a ChamQ™ Universal SYBR qPCR Master Mix kit (Tiangen, Beijing, China). Primers for detection of various genes are listed in Table 2. The following RT-PCR procedure was used: 1 min at 94 °C, 40 cycles of 10 s at 94 °C, 30 s at 55 °C, and 15 s at 68 °C. The relative abundance of the targeted mRNAs was quantified based on the cycle threshold value, which is defined as the number of cycles required to obtain a fluorescent signal above the background and was calculated according to the published method [27, 31]. To standardize the results, the relative abundance of 16S rRNA was used as an internal standard control. The relative transcriptional levels were calculated by the $-\Delta\Delta$Ct method, as follows: 2^[(Ctn$_{wt}$ − Ct0$_{wt}$) − (Ctn − Ct0)]. The Ctn and Ct0 are for the target gene and 16S rRNA in *E. coli* TWF001/pFW01-*phaCAB*, respectively; while Ctn$_{wt}$ and Ct0$_{wt}$ are for the target gene and 16 s rRNA in *E. coli* TWF001/pFW01, respectively. All assays were performed in triplicate.

Flask fermentation

The L-threonine-producing *E. coli* strains were grown on plates containing LB medium (5-g/L yeast extract, 10-g/L tryptone, and 10-g/L NaCl) for 24 h and transferred to a test tube containing 5-mL LB medium. After growing for 4 h, the OD_{600} of the culture was measured. Then, the culture was transferred to a flask-containing 25-mL LB medium, with the initial OD_{600} of 0.1. After growing for 4 h, 5-mL culture with the OD_{600} of 1.5 was transferred to a flask-containing 30-mL fermentation medium I [2-g/L yeast extract, 2-g/L citric acid, 25-g/L $(NH4)_2SO_4$, 7.46-g/L KH_2PO_4, 30-g/L glucose, 2-g/L $MgSO_4{\cdot}7\ H_2O$, 5-mg/L $FeSO_4{\cdot}7H_2O$, 5-mg/L $MnSO_4{\cdot}4\ H_2O$, and 20-g/L $CaCO_3$, pH 6.8 or pH 7.0] [17, 41, 46] and agitated for 36 h at 200 rpm.

Table 2 Primers used in this study

Primer name	Sequence (5′–3′)	Restriction site
phaCAB-F	CTGCTCGAGAGAAGGAGAATCAAATCATGGCGA CCGG	*Xho*I
phaCAB-R	CCGGAATTCAGGTCAGCCCATATGCAGG	*Eco*RI
RT-16sRNA-F	TCGGGAACCGTGAGACAGG	
RT-16sRNA-R	CCGCTGGCAACAAAGGATAAG	
RT-*aceA*-F	GTCCTGAATGCCTTTGAAC	
RT-*aceA*-R	TCGCTGTCATACGGGTCGC	
RT-*aceB*-F	CAACCGATGAACTGGCTTTC	
RT-*aceB*-R	ACGCCACAACGCAATAAACT	
RT-*aspC*-F	ACCGCATATACGCCAAACTC	
RT-*aspC*-R	GTTGCCACCATCCTGAGC	
RT-*thrA*-F	TTCCTACTTCGGCGCTAAAG	
RT-*thrA*-R	TTGCCATGTTATTCAGATTG	
RT-*thrB*-F	GCTGAACCCTACCGTGAACG	
RT-*thrB*-R	ATATGAACAAAACCTTCCTG	
RT-*thrC*-F	CGCTGGCATTTAAAGATTTC	
RT-*thrC*-R	TTGCCGCCCAATGTACAGAAC	
RT-*poxB*-F	CAGACGCTTTTTCTACACGG	
RT-*poxB*-R	CAGTAGTGCAGATGAAACTG	
RT-*pta*-F	GCTAACACCAAAGACGCTG	
RT-*pta*-R	TCGATACGCTCTTTCAGCTG	
RT-*ackA*-F	CTATTCTGGCACAAAAACCAG	
RT-*ackA*-R	GGTGCAAAAGAAGCTGCATC	
RT-*acs*-F	TCAGCACCAGGCGGAAGA	
RT-*acs*-R	ACCCGGATGATAATCAAAGAC	

The restriction enzyme sites are underlined

Fed-batch fermentation

For fed-batch fermentation, a 3-L BioFlo310 fermenter (New Brunswick Scientific, USA) was applied. L-Threonine-producing *E. coli* strains were grown on LB plates for 16 h and transferred to a 250-mL flask-containing 25-mL LB medium. After growing for 4 h at 37 °C and 200 rpm, the culture was transferred to a 500-mL flask-containing 100-mL seed medium I (32.5-g/L glucose, 5-g/L $(NH_4)_2SO_4$, 15-g/L yeast extract, 9.5-g/L KH_2PO_4, 24.35-g/L K_2HPO_4, 1-g/L $MgSO_4{\cdot}7H_2O$, pH 7.0) with the initial OD_{600} of 0.1. After growing for 4 h at 37 °C, all the seed culture was transferred to the 3-L fermenter with 800-mL fermentation medium II (20-g/L glucose, 3-g/L yeast extract, 2-g/L KH_2PO_4, 10-g/L $(NH_4)_2SO_4$, 0.5-g/L $MgSO_4{\cdot}7H_2O$, 5-mg/L $FeSO_4{\cdot}7H_2O$, and 5-mg/L $MnSO_4{\cdot}4H_2O$). The inoculum ratio was 10% (v/v) and the initial glucose concentration was 20 g/L. When glucose concentration in the medium was below 5 g/L, a feeding solution-containing 800 g/L of glucose was supplied into the medium. The incubation temperature was set at 37 °C, and the pH was controlled at 6.8 with $NH_3{\cdot}H_2O$. The dissolved oxygen was kept around 30% by adjusting the agitation speed and the aeration rate.

For larger scale fed-batch fermentation, a stirred 10-L fermenter system (PV13140, Zhenjiang Dongfang Shenggong Equipment Technology Co., Ltd.) was applied. L-Threonine-producing *E. coli* strains were grown on LB plates for 16 h and transferred to a 500-mL flask-containing 100-mL seed medium II [1.2-g/L sucrose, 10-g/L peptone, 8-g/L yeast extract, 4-g/L $(NH_4)_2SO_4$, 3-g/L K_2HPO_4, 0.4-g/L $MgSO_4$, 0.01-g/L $FeSO_4$, 0.01-g/L $MnSO_4$, 5-g/L sodium glutamate, 0.2-g/L methionine, and pH 7.0] [34]. After growing for 4 h at 37 °C and 200 rpm, the culture was transferred to two 2-L flasks, each containing 500-mL seed medium III (30-g/L glucose, 2.86-g/L yeast extract, 5.7-g/L corn syrup, 2.86-g/L K_2HPO_4, 0.57-g/L $MgSO_4$, 4.29-g/L amino acid mixture, 1-mg/L vitamin B1, 0.1-mg/L ATP, and pH 7.0) [34], and the initial OD_{600} was adjusted as 0.1. After growing for 4 h at 37 °C, all the culture was transferred to the 10-L fermenter-containing 4-L fermentation medium III (25-g/L glucose, 3.2-g/L corn syrup, 0.63-g/L betaine hydrochloride, 0.39-g/L $MgSO_4$, 0.85-g/L KCl, 10-mg/L $FeSO_4$, 10-mg/L $MnSO_4$, 0.84-g/L H_3PO_4, 1.05-g/L amino acid mixture, 24-mg/L antifoaming oil, and pH 6.8) [34]. The inoculum ratio was 10% (v/v) and the initial glucose concentration was 25 g/L. The temperature was set at 37 °C, and

the pH was controlled between 6.7 and 6.9 with $NH_3 \cdot H_2O$. The dissolved oxygen level was kept at 25–35% by agitation (200–800 rpm). The glucose concentration was kept at 5–20 g/L by feeding the concentrated glucose (600 g/L). When the dissolved oxygen level is over 30%, the glucose feeding pump was manually turned on. Sucrose was used instead of glucose in the seed medium, because sucrose was better than glucose for the growth of this *E. coli* strain according to the preliminary experiments. The addition of small amount of low-cost amino acid mixture, vitamin B1, and ATP in some media is also based on the preliminary experiments which showed that these additions could improve the cell viability for L-threonine production. Every gram amino acid mixture used in this study contains 0.026-g aspartate, 0.034-g glutamate, 0.025-g serine, 0.018-g glycine, 0.014-g threonine, 0.010-g arginine, 0.014-g alanine, 0.003-g tryptophan, 0.004-g cysteine, 0.021-g methionine, 0.011-g phenylalanine, 0.011-g isoleucine, 0.009-g leucine, and 0.00419-g lysine.

Determination of OD_{600}, amino acids, glucose, and organic acids

Biomass was determined by measuring OD_{600} with UV-1800 spectrophotometer (Shimadzu, Japan). During fermentation, 1 mL of the culture broth was taken at different time points and centrifuged at 12,000 rpm for 5 min. The supernatant was used to analyze the levels of glucose, amino acids, and organic acids.

The glucose concentration was measured with an SBA-40C biosensor (Institute of Biology, Shan-dong Academy of Science, China). To determine the levels of amino acids, the supernatant was diluted 20–100 folds, and filtered and analyzed by the 1200 series HPLC system (Agilent Technology, USA); the separation and quantification of amino acids were performed on a Thermo ODS-2HYPERSIL C18 column (250 mm × 4.0 mm, USA) using the ortho-phthalaldehyde precolumn derivatization method [14]. All the solvents used in HPLC analysis were purchased from Sigma-Aldrich (Shanghai, China).

The intracellular levels of acetyl-CoA and malate were determined according to the previous publication [41]. Two-mL *E. coli* culture (OD_{600} around 9.0) was harvested, washed with filtered water at 4 °C, and disrupted by a ultrasonic cell disruptor (Scientz-II D) for 10 min. The supernatants were collected and analyzed immediately. One unit of OD_{600} corresponds to 1.7-g/L cells. For acetyl-CoA measurement, the acetyl-CoA Assay Kit (Solarbio, Beijing, China) was used. According to the OD at 340 nm, the acetyl-CoA concentrations were calculated with the following formula: acetyl-CoA (nmol/g wet cell) = $(1640 \times \Delta A + 0.012)/(OD_{600} \times 1.7 \times 0.002)$; $\Delta A = A_{80s} - A_{20s}$.

For malate determination, the above supernatants were subjected to Agilent 1260 HPLC equipped with Diamonsil C18 column (5 μm, 250 mm × 4.6 mm no. 99603) (DiKMA technology, Beijing, China). A linear gradient elution procedure was employed as methanol:H_2O:phosphate (from 5:95:0.05 to 60:40:0.05) in 20 min. Samples were detected with an ultraviolet detector at emission wavelengths 210 nm with a flow rate of 0.8 mL/min.

Qualitative and quantitative analysis of PHB

For intracellular PHB granules analysis, cells were harvested and washed by phosphate buffer saline (pH7.4), and then prepared for further microscopic examination according to previous method [29]. One-μmol Nile Red dissolved in dimethyl sulfoxide (1 μg/μL) was added [33]. The mixtures were incubated in dark at 37 °C for 30 min after vigorous mixing. Finally, the stained cells were washed three times with deionized water to remove any residual dye and resuspended in phosphate buffer saline (pH 7.4), and 10 μL of the solution was prepared on the slides with cover glass and nail polish to visualization by fluorescence microscope (Leica TCS SP8, Leica, Germany). Cell excitation was accomplished using a 488 nm argon laser. Photographs were captured with a Leica TCS SP8 software (CellSens Standard 1.9).

Quantification of PHB was according to previous method [29]. GC-2010 plus system (Shimadzu, Japan) was employed with a DB-WAX column (30 m × 0.32 mm) (Agilent Technologies, Waldbronn, Germany) and a flame ionization detector, and the injection temperature was 250 °C. Cells were harvested by centrifugation at 10,000 rpm for 5 min, washed twice with pH 7.2 phosphate-buffered saline, and then lyophilized for 48 h. About 10-mg lyophilized cells, 2-mL methanol (with 3% H_2SO_4) and 2-mL chloroform were added to esterification tubes and treated in boiled water for 8 h. 1 mL distilled water was added to esterification tubes at room temperature, and rotary vibrated for 5 min, and then, 0.5 mL of organic phase was collected and filtrated with 0.22-μm filters (Satorious, Germany). Calibration curves were constructed with commercially available PHB (Sigma-Aldrich, Saint Louis, Missouri).

Results

L-Threonine production in *E. coli* TWF001 is enhanced by overexpression of the gene cluster *phaCAB*

Overexpression of the gene cluster *phaCAB* in L-isoleucine producing *C. glutamicum* WM001 could significantly enhance L-isoleucine production [29]. Since L-threonine is

the intermediate of L-isoleucine, the overexpression of the gene cluster *phaCAB* might also improve L-threonine production in the L-threonine-producing *E. coli* TWF001 [46]. The gene cluster *phaCAB* from *R. eutropha* [33] was overexpressed in TWF001, resulting in the strain TWF001/pFW01-*phaCAB*. The pH of the medium used for L-threonine production is usually 7.0 [17, 39], but the solubility of the fermentation medium used in this study at pH 7.0 is not as good as at pH 6.8. Therefore, the cell growth, glucose consumption, L-threonine production, and PHB formation in TWF001/pFW01-*phaCAB* grown at pH 7.0 and pH 6.8 were investigated, using TWF001/pFW01 as a control (Fig. 2). TWF001/pFW01-*phaCAB* and TWF001/pFW01 grew better, consumed glucose faster, and produced more L-threonine when grown at pH 6.8 than at pH 7.0; but TWF001/pFW01-*phaCAB* always grew better, consumed glucose faster, and produced more L-threonine than TWF001/pFW01 when they were grown at the same pH condition. After 36-h fermentation at pH 6.8, TWF001/pFW01-*phaCAB* produced 17.0-g/L L-threonine from 30-g/L glucose, and its OD_{600} reached 18.0, while TWF001/pFW01 only produced 9.7-g/L L-threonine from 29.1-g/L glucose, and its OD_{600} reached 16.0 (Fig. 2). The yield from glucose to L-threonine was 0.567 g/g for TWF001/pFW01-*phaCAB* but only 0.334 g/g for TWF001/pFW01. Compared to TWF001/pFW01, the L-threonine production and the conversion efficiency of glucose to L-threonine in TWF001/pFW01-*phaCAB* increased by 75% and 70%, respectively. The results indicate that overexpression of the gene cluster *phaCAB* significantly improved L-threonine production in *E. coli* TWF001.

Acetate accumulation in *E. coli* affects the L-threonine production, but it could be reduced by the intracellular PHB production [10, 12, 13, 24]. The levels of acetate accumulation in TWF001/pFW01-*phaCAB* and TWF001/pFW01 after 36-h fermentation (pH 6.8) were determined (Fig. 2d). TWF001/pFW01-*phaCAB* accumulated 2.83-g/L acetate, while TWF001/pFW01 accumulated 7.65-g/L acetate. This suggests that PHB biosynthesis could decrease the intracellular level of acetate, and the increased L-threonine production in TWF001/pFW01-*phaCAB* might be relevant to the decrease of intracellular acetate.

To investigate whether the overexpression of *phaCAB* in TWF001 could produce PHB, TWF001/pFW01-*phaCAB* and TWF001/pFW01 cells were stained with Nile Red and analyzed using fluorescence microscopy (Fig. 3). In the

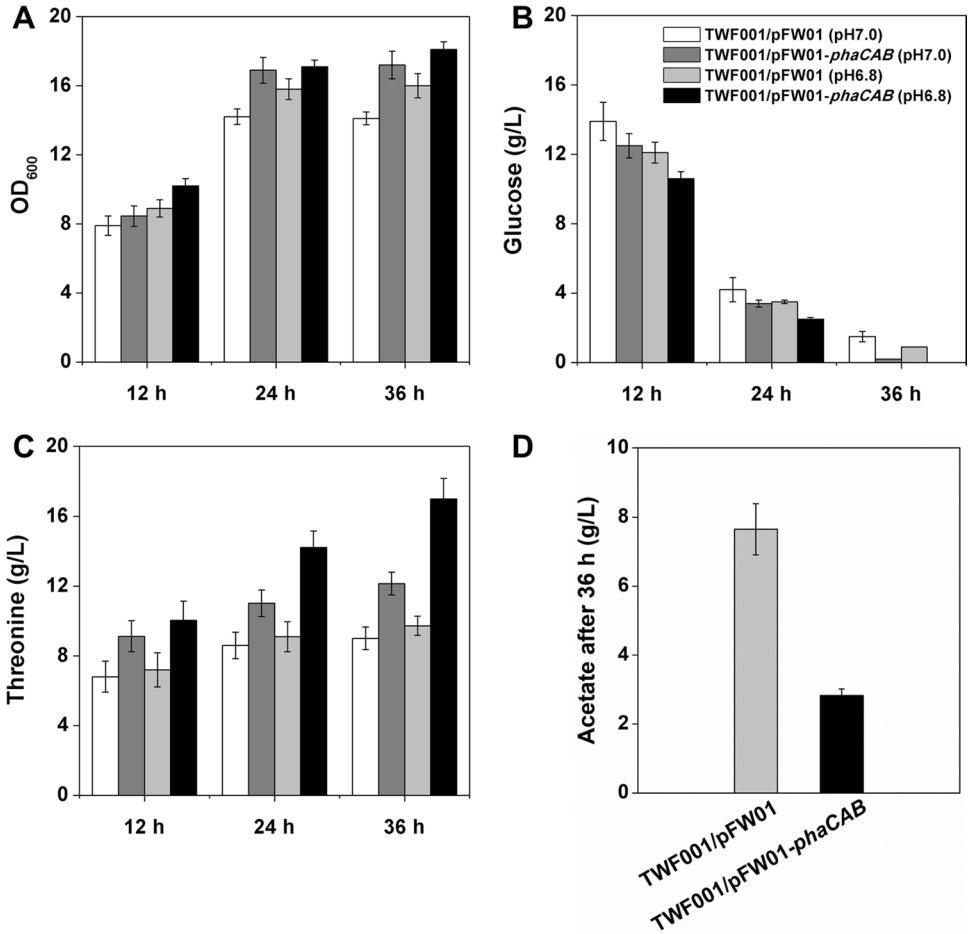

Fig. 2 Comparison of flask fermentation of *E. coli* TWF001/pFW01-*phaCAB* and TWF001/pFW01 grown at pH 7.0 and pH 6.8. **a** Cell growth; **b** glucose consumption; **c** L-threonine production. **d** The extracellular levels of acetate accumulated by *E. coli* TWF001/pFW01-*phaCAB* and TWF001/pFW01 cells after 36-h flask fermentation. The error bars indicate the standard deviations from three independent experiments

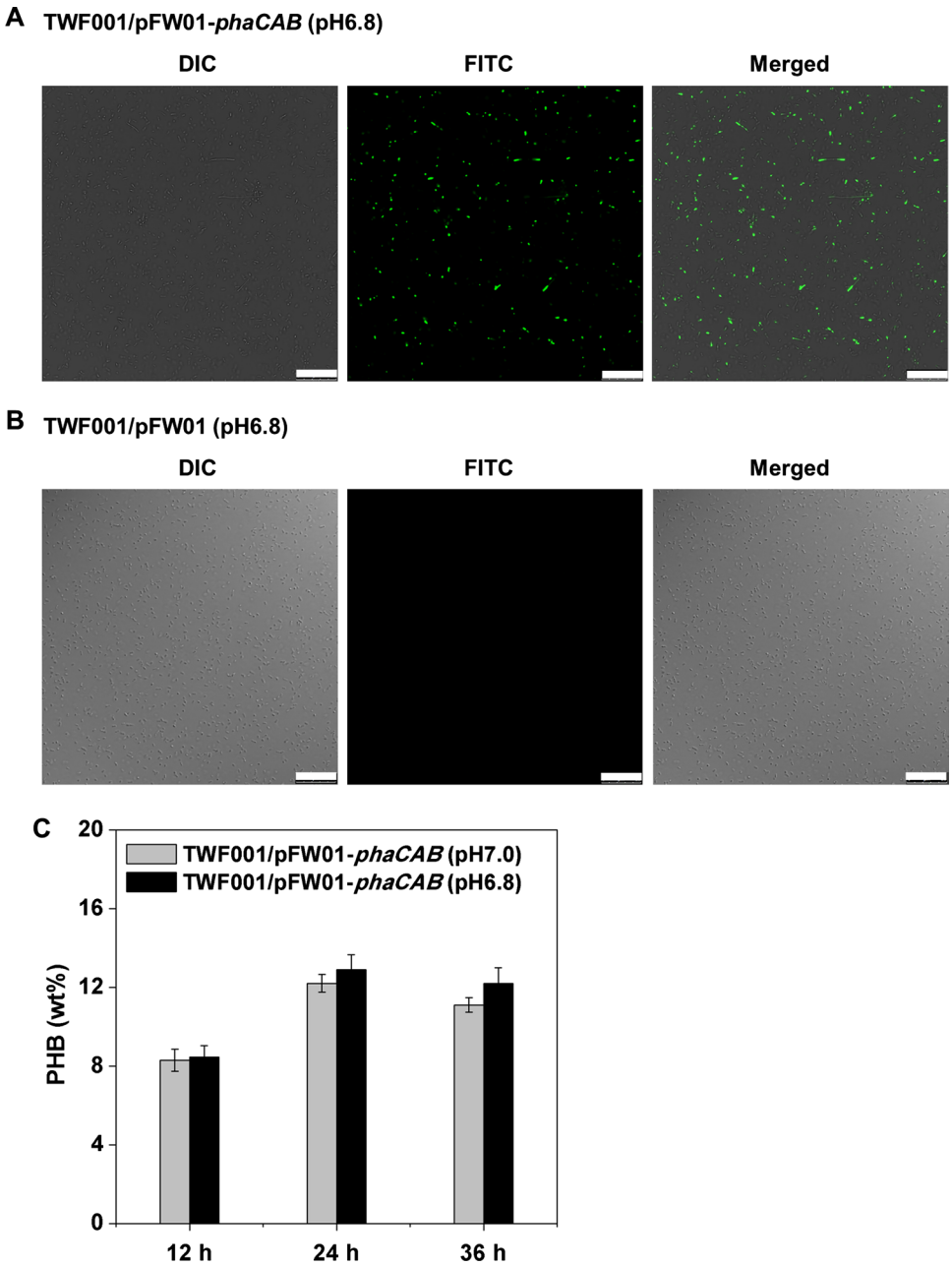

Fig. 3 PHB accumulation determination. **a** PHB accumulation in *E. coli* TWF001/pFW01-*phaCAB* and TWF001/pFW01 grown at pH 7.0 and pH 6.8. **b** Fluorescence microscopy images of TWF001/pFW01-*phaCAB* cells grown at pH 6.8. **c** Fluorescence microscopy images of TWF001/pFW01 cells grown at pH 6.8. *DIC* differential interference contrast, *FITC* fluorescence contrast, *Merged* overlapped contrast of DIC and FITC. Scale bar in white is 25 μm

DIC mode, some long cells were observed in TWF001/pFW01-*phaCAB* (Fig. 3a), but not in TWF001/pFW01 cells (Fig. 3b). In the FITC mode, green particles were observed in TWF001/pFW01-*phaCAB* (Fig. 3a), but not in TWF001/pFW01 cells (Fig. 3b). This indicates that PHB was produced in TWF001/pFW01-*phaCAB* cells due to the overexpression of the gene cluster *phaCAB,* because Nile Red-stained PHB granules can emit green fluorescence. When the DIC and FITC spectra were merged, it can be seen that only part of the TWF001/pFW01-*phaCAB* cells produced PHB and most of these cells were larger than the ones without PHB production. The formation of intracellular PHB granules depends on cell physiology, carbon source, nitrogen source, and the plasmid stability. More L-threonine production could be expected if the heterogeneity for PHB production in cells can be resolved and all cells could produce PHB. The PHB production in TWF001/pFW01-*phaCAB* grown at pH 7.0 and pH 6.8 was also quantified (Fig. 3c). TWF001/pFW01-*phaCAB* cells grown at pH 6.8 could produce 12.9% and 12.2% (wt%) PHB after 24-h and 36-h fermentation, respectively. However, more L-threonine was produced in TWF001/pFW01-*phaCAB* after 36-h fermentation than after 24 h fermentation (Fig. 2c). The results indicate that overexpression of the gene cluster *phaCAB* in *E. coli* TWF001 could produce PHB at the early growth stage and significantly improved L-threonine production at the latter stage.

Acetyl-CoA and malate biosynthesis increased in *E. coli* TWF001 after overexpression of *phaCAB*

To better understand the influence of the *phaCAB* overexpression in *E. coli* on the L-threonine biosynthesis, the levels of a few key intermediate metabolites and by-product acetate as well as the relative transcriptional levels of relevant genes were determined. Acetyl-CoA is the precursor of PHB, and can be catalyzed by malate synthase encoded by *aceA* to form malate and then fluxing to the biosynthesis of L-threonine. The level of acetyl-CoA is a key factor for both PHB and L-threonine synthesis. The intracellular levels of acetyl-CoA and malate in TWF001/pFW01 and TWF001/pFW01-*phaCAB* grown at the log phase were determined (Fig. 4a). The levels of acetyl-CoA and malate were both increased in TWF001/pFW01-*phaCAB*. The level of acetyl-CoA reached to 4.51 μmol/g, which is 2.16 times higher than that of the control TWF001/pFW01. The level of malate in TWF001/pFW01-*phaCAB* increased by 43%, compared with the control TWF001/pFW01. The results indicate that the overexpression of *phaCAB* could contribute to the enhancement of carbon flux to the acetyl-CoA and malate, further enhancing L-threonine production.

In TWF001/pFW01-*phaCAB*, acetyl-CoA could be used to biosynthesize PHB, acetate, and also flux to glyoxylate shunt. The enhanced glyoxylate shunt and the decreased acetate level are favorable for L-threonine biosynthesis. As expected, acetate accumulation could be reduced in TWF001/pFW01-*phaCAB* [10, 12, 13, 24]. In *E. coli*, acetate was synthesized either from pyruvate by PoxB or from acetyl-CoA by Pta-AckA; acetate could also be consumed to form acetyl-CoA by acetyl-CoA synthetase encoded by *acs* (Fig. 1). RT-PCR analysis showed that the transcriptional levels of *poxB*, *pta,* and *ackA* were down-regulated, but the transcriptional level of *acs* was up-regulated in TWF001/pFW01-*phaCAB* cells grown at the mid-log phase, compared to that in TWF001/pFW01 cells (Fig. 4b). In addition, the transcriptional levels of *aceA*, *aceB*, *aspC*, *thrA*, *thrB,* and *thrC* in TWF001/pFW01-*phaCAB* increased, compared to that in TWF001/pFW01 (Fig. 4b). This suggests that PHB formation could influence carbon flow distribution and enhance the glyoxylate shunt pathway, leading more carbon flux into acetyl-CoA and malate and eventually L-threonine production.

L-Threonine production was significantly increased TWF001/pFW01-*phaCAB* cells in fed-batch fermentation

The L-threonine production in the fed-batch fermentation of TWF001/pFW01-*phaCAB* was investigated using a 3-L fermenter (Fig. 5). TWF001/pFW01-*phaCAB* cells grew better than TWF001/pFW01. TWF001/pFW01-*phaCAB* reached the maximum OD_{600} of 49.7 at 28 h, while TWF001/pFW01 reached the maximum OD_{600} of 35.2 at 20 h (Fig. 5a). PHB granules in TWF001/pFW01-*phaCAB* might contribute to the high OD_{600}. Similar patterns of glucose consumption were observed for TWF001/pFW01 and TWF001/pFW01-*phaCAB* during the fermentation (Fig. 5b). Similar levels of L-threonine production were observed in TWF001/pFW01-*phaCAB* and TWF001/pFW01 at the first 12 h, and then, more L-threonine were produced in TWF001/pFW01-*phaCAB* than in TWF001/pFW01. After 44 h, 96.4-g/L L-threonine were produced in TWF001/pFW01-*phaCAB*, but only 73.2-g/L L-threonine were produced in TWF001/pFW01 (Fig. 5c). The conversion ratio of glucose to L-threonine reached 0.54 g/g in TWF001/pFW01-*phaCAB*, but only reached 0.41 g/g in TWF001/pFW01. TWF001/pFW01-*phaCAB* produced 11.5% PHB and 6.3-g/L acetate, while TWF001/pFW01 produced 14.8-g/L acetate.

To further investigate the L-threonine production in larger scale fed-batch fermentation, *E. coli* strains were fermented in 10-L fermenter (Fig. 6). TWF001/pFW01-*phaCAB* cells grew slightly slower than TWF001/pFW01 before 6 h, but faster afterwards. After 36-h fermentation, TWF001/pFW01-*phaCAB* reached the maximum OD_{600} of 41.6, while TWF001/pFW01 reached the maximum OD_{600} of 33.2 (Fig. 6a). Much more L-threonine production was produced in TWF001/pFW01-*phaCAB* during the whole fermentation.

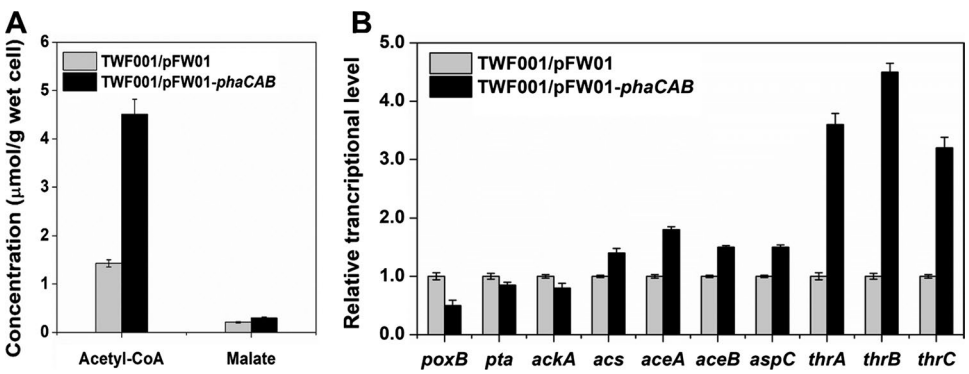

Fig. 4 **a** The intracellular levels of acetyl-CoA and malate in *E. coli* TWF001/pFW01-*phaCAB* and TWF001/pFW01 grown at the log phase; **b** relative transcription levels of *poxB*, *pta*, *ackA*, *acs*, *aceA*, *aceB aspC*, *thrA*, *thrB,* and *thrC* analyzed by RT-PCR. The error bars indicate the standard deviations from three independent experiments

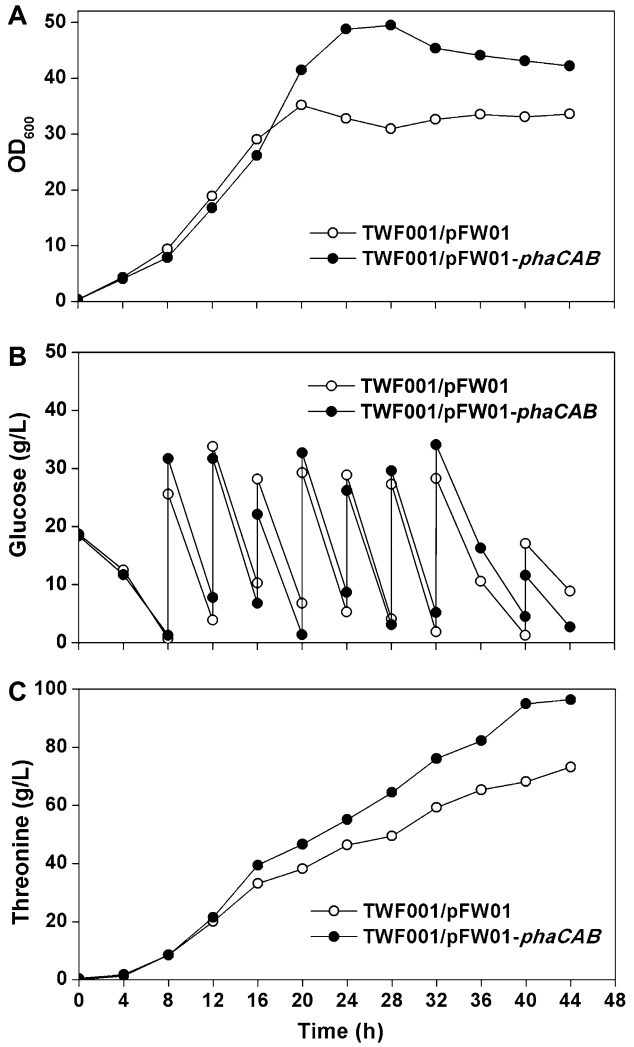

Fig. 5 Fed-batch fermentation profiles of *E. coli* TWF001/pFW01-*phaCAB* and TWF001/pFW01 in 3-L fermenter. **a** OD_{600}; **b** residual glucose; **c** L-threonine concentration

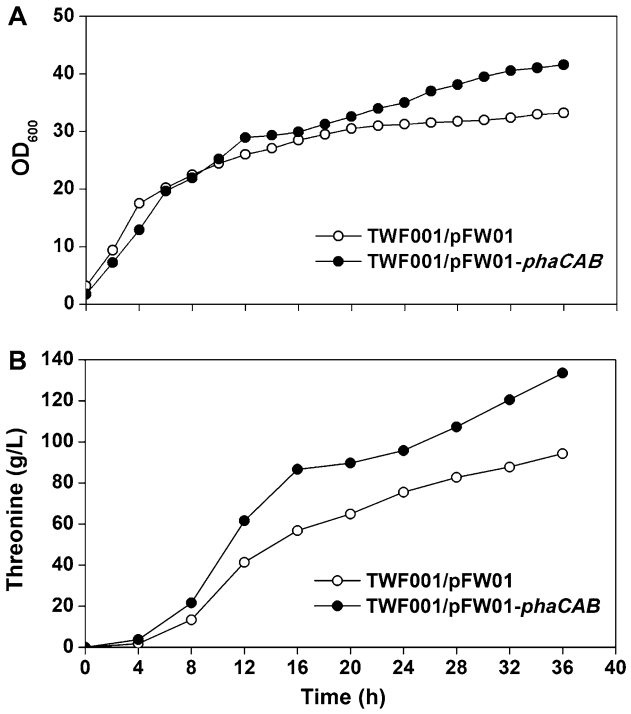

Fig. 6 Fed-batch fermentation profiles of *E. coli* TWF001/pFW01-*phaCAB* and TWF001/pFW01 in 10-L fermenter. **a** OD_{600}; **b** L-threonine concentration

After 36 h, 133.5-g/L L-threonine was produced in TWF001/pFW01-*phaCAB*, but only 94.3-g/L L-threonine was produced in TWF001/pFW01 (Fig. 6b). The conversion efficiency of glucose to L-threonine in TWF001/pFW01-*phaCAB* reached 0.50 g/g, while it only reached 0.38 g/g in the control TWF001/pFW01. In addition, TWF001/pFW01-*phaCAB* cells also accumulated approximately 13.0% PHB after 36-h fermentation.

In recent years, fermentation optimization has also been proved effective in improving L-threonine production. L-Threonine production in *E. coli* EC125 under the optimal feeding control reached 105.3 g/L [25], L-threonine production in *E. coli* TRFC with pseudo-exponential glucose-start feeding strategy reached 124.6 g/L [36], and L-threonine production in *E. coli* JLTHR reached 127.3 g/L when fed with 2-g/L betaine hydrochloride in 5-L fermenter [34]. In this study, 133.5-g/L L-threonine was produced in *E. coli* TWF001 when overexpressing *phaCAB*, strongly suggesting the importance of *phaCAB* expression. The PHB accumulation in TWF001/pFW01-*phaCAB* might rebalance metabolic flux distribution, decrease the acetate level, and increase the acetyl-CoA and malate levels [29], leading to the enhanced glyoxylate shunt and the increase of L-threonine production. The results in this study suggest that expressing the gene cluster *phaCAB* is an efficient strategy to improve L-threonine production in *E. coli*.

Discussion

In this study, the gene cluster *phaCAB* were introduced into the L-threonine-producing strain TWF001. The recombinant strain TWF001/pFW01-*phaCAB* produced 133.5 g/L after 36-h fed-batch fermentation in 10-L fermenter. Table 3 shows the comparison of production of L-threonine, acetyl-CoA, acetate, and PHB in TWF001/pFW01-*phaCAB* and TWF001/pFW01. The results suggest that the PHB accumulation could enhance the L-threonine production, possibly because the metabolic flux distribution was rebalanced [10, 12, 13, 44].

Our previous studies showed that enhancing the glyoxylate shunt [41, 46] and reducing the acetate level [5] could significantly improve the L-threonine production.

Table 3 Flask fermentation and fed-batch fermentation of TWF001/pFW01-*phaCAB* and TWF001/pFW01 strains

	OD_{600}	L-Threonine (g/L)	Yield on glucose (g/g)	Acetyl-CoA (μmol/g)	Acetate (g/L)	PHB (wt%)
Flask fermentation						
TWF001/pFW01-*phaCAB*	18.0	17.0	0.57	1.43	2.83	12.2
TWF001/pFW01	16.0	9.72	0.33	4.51	7.65	–
Fed-batch fermentation in 3-L fermenter						
TWF001/pFW01-*phaCAB*	42.2	96.4	0.54	–	6.3	11.5
TWF001/pFW01	33.6	73.2	0.41	–	14.8	–
Fed-batch fermentation in 10-L fermenter						
TWF001/pFW01-*phaCAB*	41.6	133.5	0.50	–	–	13.0
TWF001/pFW01	33.2	94.3	0.38	–	–	–

The sufficient supply of precursor malate and acetyl-CoA is important for the glyoxylate shunt and L-threonine overproduction in *E. coli* [41]. Acetyl-CoA is mainly derived from pyruvate. If the glyoxylate shunt operates actively and acetyl-CoA is actively used for the production of oxaloacetate, L-threonine production might be enhanced [23, 34]. PHB accumulation might rebalance the metabolic flux [31]. Overexpression of *phaCAB* could pull more metabolic flux to acetyl-CoA [38], resulting in a larger "acetyl-CoA pool" [29]. Consequently, the carbon flux to the malate was also increased 43%, the increased levels of acetyl-CoA and malate could further lead to the enhancement of glyoxylate shunt with the up-regulation of *aceA* and *aceB*. These all benefit the improvement of L-threonine production with the up-regulated *aspC*, *thrA*, *thrB,* and *thrC* transcriptional levels. In our previous study, overexpression of *phaCAB* in *C. glutamicum* WM001 could also significantly enhance the production of L-isoleucine, the downstream product of L-threonine [29]. These results showed that PHB biosynthesis in bacteria could enhance the biosynthesis of acetyl-CoA which benefits the L-threonine accumulation.

Acetate is a major by-product for L-threonine production in *E. coli* [23, 39]. Acetate accumulation consumes a large amount of pyruvate and acetyl-CoA, and thus affects the L-threonine production [23, 39]. Reducing acetate accumulation could enhance the L-threonine production and improve glucose conversion efficiency [23, 34, 39]. Overexpressing the gene cluster *phaCAB* has been used to reduce acetate accumulation and increase the production of L-glutamate in *C. glutamicum* [24], L-tryptophan in *E. coli* [10], and succinate in *E. coli* [13]. In addition, PHB biosynthesis pathway also directly exerts metabolic control over the carbon flux distribution [35]. The L-threonine-producing *E. coli* strain TWF001 could accumulate a large amount of acetate (7.65 g/L), and therefore, in this study, the gene cluster *phaCAB* was overexpressed in TWF001. The resulting strain TWF001/pFW01-*phaCAB* only accumulated 2.83-g/L acetate, but produced 216% more acetyl-CoA and 43% more malate. All these influences could benefit the enhancement of L-threonine production. The genes *poxB*, *pta,* and *ackA* were down-regulated, while the gene *acs* was up-regulated, suggesting that the carbon flux was directed to the production of PHB or L-threonine. In addition, PHB is an inclusion body and could improve the resistance of bacterial cells to the harsh environment [9], warrant normal carbon metabolism, and enhance the carbon metabolism, leading to more carbon flow to acetyl-CoA [3, 37, 47], and up-regulate the expression of GroEL, GroES, DnaK, and sigma 38 factor [4, 11]. Therefore, the influence of PHB accumulation on L-threonine production in *E. coli* TWF001 might be complex.

Conclusions

In this study, the PHB synthesis pathway was introduced into an L-threonine-producing *E. coli* strain TWF001, leading to a significant increase of L-threonine production as well as the PHB formation. The L-threonine production of TWF001/pFW01-*phaCAB* reached 133.5 g/L after fed-batch fermentation in 10-L fermenter. Transcription analysis showed that the key genes in the L-threonine biosynthetic pathway were up-regulated, the genes *poxB*, *pta,* and *ackA* relevant to the formation of acetate were down-regulated, and the gene *acs* encoding the enzyme which converts acetate to acetyl-CoA was up-regulated, further leading to the increased levels of acetyl-CoA and malate, while the decreased acetate accumulation. The results suggested that PHB formation in *E. coli* could enhance the carbon flux towards L-threonine production.

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