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Research article

Betaine supplementation improved L-threonine fermentation of *Escherichia coli* THRD by upregulating *zwf* (glucose-6-phosphate dehydrogenase) expression

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ABSTRACT

Background: The supplementation of betaine, an osmoprotective compatible solute, in the cultivation media has been widely used to protect bacterial cells. To explore the effects of betaine addition on industrial fermentation, *Escherichia coli* THRD, an L-threonine producer, was used to examine the production of L-threonine with betaine supplementation and the underlying mechanism through which betaine functions was investigated.
Results: Betaine supplementation in the medium of *E. coli* THRD significantly improved L-threonine fermentation parameters. The transcription of *zwf* and corresponding enzyme activity of glucose-6-phosphate dehydrogenase were significantly promoted by betaine addition, which contributed to an enhanced expression of *zwf* that provided more nicotinamide adenine dinucleotide phosphate (NADPH) for L-threonine synthesis. In addition, as a result of the betaine addition, the betaine-stimulated expression of enhanced green fluorescent protein (eGFP) under the *zwf* promoter within a plasmid-based cassette proved to be a transcription-level response of *zwf*. Finally, the promoter of the phosphoenolpyruvate carboxylase gene *ppc* in THRD was replaced with that of *zwf*, while L-threonine fermentation of the new strain was promoted by betaine addition.
Conclusions: We reveal a novel mode of betaine that facilitates the microbial production of useful compounds. Betaine supplementation upregulates the expression of *zwf* and increases the NADPH synthesis, which may be beneficial for the cell growth and thereby promote the production of L-threonine. This finding might be useful for the production of NADPH-dependent amino acids and derivatives in *E. coli* THRD or other *E. coli* strains.
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1. Introduction

L-Threonine is an essential amino acid that is widely used in animal feeds, human foods, and pharmaceutical and cosmetics products [1]. L-Threonine is currently produced mainly by *Escherichia coli* fermentation, which has undergone substantial improvements driven by strain engineering and process optimization. Intensive studies have reported on metabolic engineering of L-threonine biosynthesis in *E. coli*, e.g., overexpressing the key enzymes resistant to feedback inhibition in condensing carbon influx, weakening the competing branches, reducing the intracellular consumption, and enhancing the secretion of L-threonine [2]. Meanwhile, the importance of carbon and nitrogen sources, feed media, iron, oxygen, and growth factor biotin was identified, and the corresponding feeding strategies were optimized to increase L-threonine production [3]. In a previous study, we supplemented betaine in the medium of an industrial producer *E. coli* THRD in an attempt to relieve the osmotic stress caused by a high substrate or product concentration as well as the massive addition of neutralizing agent during L-threonine fermentation, and found that L-threonine production was significantly improved (unpublished).

The addition of betaine (*N*,*N*,*N*-trimethylglycine), a major compatible solute, has been successfully used in the fields of applied microbiology and biotechnology because of its special methylation-related structure [4]. However, the underlying mechanisms through which betaine functions are complicated and far from being fully elucidated.

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Nevertheless, several aspects can be proposed based on a survey of published studies: (i) Betaine is a well-known osmoprotectant for various strains such as *E. coli* [5], *Bacillus subtilis* [6], and *Gluconacetobacter diazotrophicus* [7]; (ii) in addition to protecting whole cells, betaine functions as a protein (enzyme) protector [8,9]; (iii) betaine has been used as the methyl group donor for the production of methionine, vitamin B_{12}, and natural compounds that require SAM-mediated methylation steps; (iv) betaine can improve cell permeability as a surfactant; and (v) interestingly, a recent study revealed that betaine addition was able to prevent glucose caramelization during autoclaving [10].

Here, we reveal a novel mode of betaine that facilitates the microbial production of useful compounds. That is, betaine supplementation upregulated the expression of *zwf*, which encodes glucose-6-phosphate dehydrogenase (G6PDH), thereby enabling *E. coli* THRD to produce more L-threonine due to an enhanced nicotinamide adenine dinucleotide phosphate (NADPH) supply. The application potential of this finding was proved and highlighted.

2. Materials and methods

2.1. Strains

The bacterial strains, plasmids, and primers used in this study are listed in supplementary Table S1. The L-threonine producer *E. coli* THRD [11,12], which originated from the repeated mutagenesis of MG1655, has been deposited in China General Microbiological Culture Collection Center (Registration number 11074, undisclosed). THRD, with leaky synthesis of L-isoleucine (ILE^L), is resistant to L-threonine analog α-amino-β-hydroxyvaleric acid (AHV^r). Although the genomic background has not been well understood, we found two mutations potentially responsible for these genetic markers. The mutation within *thrA* (NCBI Gene ID: 945803) encoding bifunctional enzyme aspartate kinase/homoserine dehydrogenase is G1297A (glycine433argine), which is a common mutation for releasing feedback inhibition by L-threonine, and thus revealing AHV resistant [13]. The other mutation, C992G (glycine331alanine) within *tdh* (Gene ID: 948139) encoding threonine dehydratase, might be the reason for the leaky synthesis of L-isoleucine.

The strains THRDΔ*zwf* and THPZ (replacement of P*ppc* with P*zwf*) were constructed using the λ-Red- and FLP-mediated recombination methods, respectively [14]. In detail, upstream and downstream regions of *ppc* promoter were obtained by PCR using the primer pair P9 (5′-CAACTGGTTGAAGTGGTTGAGAA-3′) and P10 (5′-TACACAATC GCTCAATCACTGTCGGTCGGATAAGATG-3′), and P15 (5′-CTTAAGGAG AATGACATATGAACGAACAATATTCCGCAT-3′) and P16 (5′-ATTTCGGTT GGGTGAGCCGTGAG-3′), respectively. The Cm^r gene (approximately 1000 bp in length) was obtained using the primer pair P11 and P12, with helper plasmid pKD3 as template. The promoter sequence of *zwf* (485 bp) was amplified using the primer pair P13 (5′-GTGCGTTACAT CCCTTCGGTTCGCTAACATTGGCTTC-3′) and P14 (5′-ATTGTTCGTTCATA TGTCATTCTCCTTAAGTTAACTAACCCG-3′), with genomic DNA of THRD as template. Gene splicing by overlap extension polymerase chain reaction (SOE PCR) was used to fuse the fragments upstream and downstream of the P*ppc*, Cm^r gene, and P*zwf* with the primer pair P9 and P10. The ligation of P*zwf* within the upstream and downstream sequences of P*ppc* led to the deletion of *ppc* promoter (364 bp). To remove the Cm^r gene from the integrated locus, cells were transformed with plasmid pCP20 carrying the FLP recombinant gene. Mutant sequences were verified by PCR and subsequent sequencing.

Fragments of P_{zwf} or P_{gapA} were amplified from THRD genomic DNA, joined with *egfp* from pEGFP-N1 using SOE PCR, and finally homologously recombined with the linearized pUC19 by reverse PCR to obtain pUC-P_{zwf}-e*gfp* and pUC-P_{gapA}-e*gfp*, respectively. The two plasmids were electroporated into THRD or DH5α cells.

2.2. Growth media

The seed medium contained the following components (per liter): 40 g glucose, 6 g yeast extract, 6 g peptone, 10 mL corn steep liquor, 1.2 g KH_2PO_4, 3 g $(NH4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, and 10 mg $MnSO_4 \cdot H_2O$.

The fermentation medium contained (per liter) the following: 30 g glucose, 6 g yeast extract, 6 g peptone, 15 mL corn steep liquor, 0.5 g sodium citrate, 2 g KH_2PO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 50 mg $FeSO_4 \cdot 7H_2O$, 50 mg $MnSO_4 \cdot H_2O$, and 2–3 drops of defoamer.

2.3. Culturing conditions

Seed cultures were prepared by transferring all cells grown overnight on three agar slants into a 5 L fermenter (Shanghai Baoxing Bio-engineering Equipment Co., Ltd., Shanghai, China) containing 2 L of seed medium. The pH was maintained at 7.0 by the automated addition of NH_4OH (25%, v/v). Dissolved oxygen was maintained at a concentration >20% by varying the stirrer speed and the aeration rate. The temperature was maintained at 36°C. The seed cultures were continued until the cells grew to an optical density of 12–14 at 600 nm, while 600 mL of the culture broth was retained for the fed-batch cultures.

Fed-batch cultures were grown in a 5 L bioreactor containing 3.5 L of medium. The pH was maintained at 7.0 by the automated addition of NH_4OH (25%, v/v). Dissolved oxygen was maintained at approximately 30% by circulating 2–9 L/min of air and automatically increasing the agitation speed from 100 to 500 rpm. The temperature was maintained at 36°C. The residual glucose in the medium was quickly detected using a biosensor analyzer (SBA-40E, Institute of Biology, Shandong Academy of Sciences, Jinan, China). Sterilized 80% glucose solution was fed at appropriate rates once the glucose was exhausted. The feeding rate was controlled in response to the consumption rate to maintain the glucose concentration below 5 g/L. Betaine was first supplemented in the initial medium to a final concentration of 0.5 g/L and then fed successively with the glucose solution (containing 1 g/L betaine) when necessary.

The analysis of enhanced green fluorescent protein (eGFP) expression driven by P_{zwf} in THRD was conducted in shake flask cultures. The cells were inoculated from the activated agar slant into 30 mL of seed medium and cultivated at 36°C and 220 rpm in a rotary shaker. After 10 h, 3 mL of the seed culture was transferred into 30 mL of the fermentation medium in a 500-mL baffled flask, and the culture pH was controlled artificially at 7.0 using 25% (*w*/*v*) $NH_3 \cdot H_2O$. The residual glucose in the medium was detected using SBA-40E biosensor analyzer, and 1 mL of 60% glucose was added when the residual glucose concentration was below 5 g/L. Glucose was added approximately 3–5 times during each culture. For the betaine addition batches, glucose solution containing 1 g/L betaine was used instead.

DH5α/pUC-P_{zwf}-e*gfp* and DH5α/pUC-P_{gapA}-e*gfp* were also cultured in LB medium to demonstrate the specificity of transcriptional response to the betaine addition. The cells were inoculated from the test tube culture into 30 mL of LB medium, containing different concentrations of betaine, and cultivated at 36°C and 220 rpm in a rotary shaker.

For all the cultures, samples were taken every 2 h for the analytical procedures. All experiments were performed in triplicate.

2.4. Reverse transcription quantitative PCR analysis of zwf

A reverse transcription quantitative PCR analysis of *zwf* was conducted using a set of kits purchased from Takara Bio Inc. (Dalian, China). The samples were harvested, and the total RNA was extracted using RNAiso plus. To avoid DNA contamination, the total RNA samples were treated with gDNA Eraser at 42°C for 5 min according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent Kit following the

manufacturer's protocol. The quantitative PCR was performed by a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA) using a SYBR Premix Ex Taq™ II kit with P22 and P23 primer pair (Table S1) for the amplification of an 88-bp amplicon. The data were analyzed by the $2_T^{-\Delta\Delta C}$ method [15]. The relative abundance of 16S RNA was used as the internal standard.

2.5. Measurement of G6PDH activity

The enzyme activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured using a Glucose-6-Phosphate Dehydrogenase Kit (Comin Biotechnology Co., Ltd., Suzhou, China) following the manufacturer's instructions. Cells were disrupted by sonication using an ultrasonic disruptor (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). Cell debris was removed by centrifugation (8000 × *g* for 10 min at 4°C), and the crude cell extracts were used for the enzyme assays. Enzyme activity was measured spectrophotometrically at 340 nm. One unit of enzyme activity (U) of G6PDH was defined as the amount of enzyme releasing 1 μmol of NADPH per minute. Protein concentration was determined using a Protein Quantitative Kit (BCA) (Applygen Technologies Inc., Beijing, China) with bovine serum albumin as a standard. The specific enzyme activity was calculated as units per milligram of protein.

2.6. NADPH assay

The content of NADPH was determined using the coenzyme II NADP (H) Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Samples were taken at 6, 12, 18, and 24 h from the 5 L fed-batch fermentation medium. The absorbance was measured at 570 nm.

2.7. Detection of eGFP florescence

The florescence of eGFP was detected using an F-7000 fluorescence spectrophotometer (Hitachi, Japan) with samples diluted to an OD_{600} of 0.2–0.8. The excitation and emission wavelengths were set to 491 nm and 511 nm, respectively.

2.8. Biomass, glucose, and L-threonine determination

Cell growth was monitored by measuring OD_{600} using a 752 UV spectrophotometer, and the biomass was expressed as dry cell weight (DCW).

Glucose was determined by high-performance liquid chromatography (1200 series, Agilent Technologies, USA) equipped with an Aminex HPX-87H column (7.8 × 300 mm, Bio-Rad) and a G1362A refractive index detector (RID) under the following conditions: mobile phase 5 mM H_2SO_4, flow rate 0.6 mL/min, and column temperature 65°C.

L-Threonine was quantified by high-performance liquid chromatography involving a pre-column derivatization. The samples were diluted and derivatized with 2,4-dinitrofluorobenzene and measured by high-performance liquid chromatography with an Agilent ZORBAX Eclipse AAA column (4.6 mm × 150 mm, 5 μm). Elution was performed using a gradient of reagent A (50% acetonitrile *v*/v) and reagent B (0.05 M CH_3COONa, pH 6.4) at a flow rate of 1.0 mL/min. Ultraviolet absorption was measured at 360 nm, and the column temperature was maintained at 33°C.

2.9. Statistical analysis

All experiments were performed at least in triplicate, and statistical significance was determined via a one-way analysis of variance followed by Dunnett's multiple comparison test; statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Effects of betaine supplementation on L-threonine fermentation

To investigate the effects of betaine addition on L-threonine fermentation, we performed a 5 L fed-batch fermentation of THRD, during which a final concentration of 0.5 g/L betaine was supplemented in the initial medium, followed by the subsequent feeding of glucose solution containing 1 g/L betaine as we found that betaine with a concentration >1 g/L impaired cell growth (unpublished). The control group did not receive betaine. As shown in Fig. 1A, the maximum biomass of THRD cultured with betaine addition was 17.7 g DCW per liter, whereas the value without the betaine addition was 16.7 g/L, indicating betaine clearly has a growth stimulating effect. The total glucose consumption of THRD with betaine addition was comparable to that of the control (Fig. 1B). The final L-threonine titer at 24 h of fermentation supplemented with betaine was 13.3% higher than that without the betaine addition (Fig. 1C). Fig. 1D presents the L-threonine yields and specific titers; the yield with betaine addition was 8.7% higher than that of the control, while there was no significant difference in terms of the specific titer. The increased L-threonine yield is because of the higher biomass. Taken together, these results display the positive effects of betaine supplementation on L-threonine fermentation, while the L-threonine titer reached 117.1 g/L with a productivity of 4.88 g/L/h and yield of 0.51 g/g glucose. The promotion of L-threonine production by betaine addition is probably an effect of the increase in biomass accumulation.

3.2. Increase of zwf transcription and G6PDH activity resulting from betaine supplementation

We hypothesized gene expression regulations are involved in the alteration of central carbon metabolisms of THRD with betaine supplementation and thus determined the transcript levels of several key genes such as *pfkA*, *zwf*, and *pykF*, each using its corresponding level without betaine addition as reference. The *pfkA* gene encodes 6-phosphofructokinase I, which catalyzes the irreversible reaction of fructose-6-phosphate phosphorylation in glycolysis. The *zwf* encodes G6PDH, catalyzing the first step of the pentose phosphate pathway (PPP), which serves as a main source of reducing power (i.e., NADPH) for anabolic reactions in the cell. The *pykF* encodes pyruvate kinase I that converts phosphoenolpyruvate (PEP) to pyruvate, and the reactions in the node of PEP, pyruvate, and oxaloacetate are of great importance for L-threonine synthesis. Samples were taken at 6, 12, and 18 h from the 5 L fed-batch fermentation media, and the results showed that *zwf* transcription was remarkably regulated by betaine, which achieved 3.6-fold levels compared with the reference at 18 h (Fig. 2A). The transcript levels of *pfkA* and *pykF* did not show significant variations in response to betaine supplementation (Fig. S1). At each time point within the fermentation process, the G6PDH activity increased significantly as a result of betaine addition (Fig. 2B). At 18 h, the G6PDH activity with betaine addition reached 158 ± 5.2 U/mg proteins, approximately 3.4-fold of that of the control, a finding that was consistent with the transcriptional difference. From Fig. 2, it can be concluded that the *zwf* transcript levels and the G6PDH activities show strong growth phase-dependent effects.

3.3. Enhanced production of NADPH by betaine supplementation

The relative contribution of PPP and glycolysis to glucose catabolic flux is of great importance, partially because of the relevance to NADPH production [16]. The improved *zwf* expression due to the betaine addition may expectedly increase the NADPH supply, which is beneficial for L-threonine synthesis because 3 mol of NADPH is required to produce 1 mol of product, with aspartyl semialdehyde dehydrogenase and homoserine dehydrogenase involved [2]. Thus, we

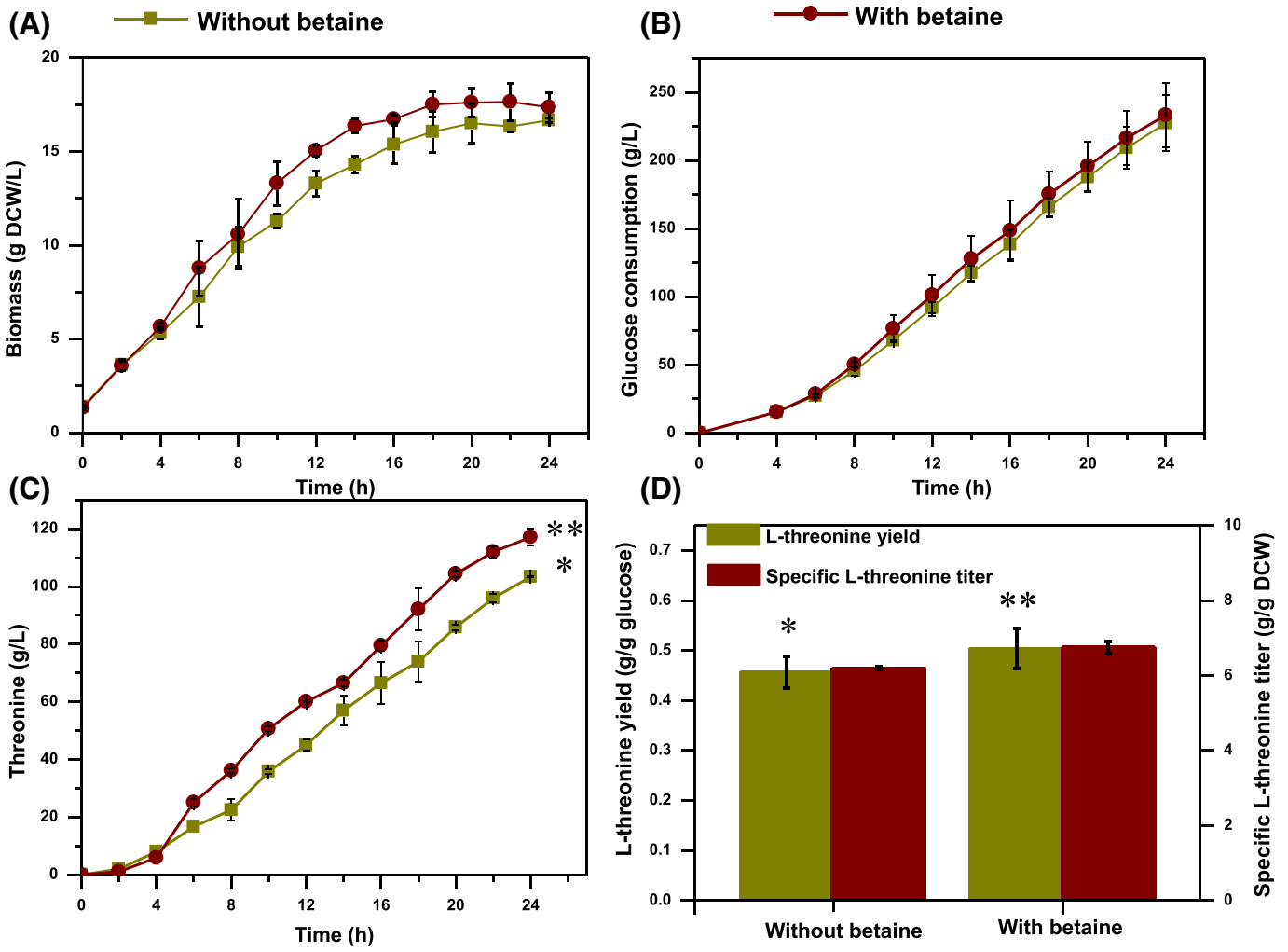

Fig. 1. Profiles of fed-batch fermentation of L-threonine by *Escherichia coli* THRD in a 5 L fermenter with or without betaine supplementation. (A) Biomass, (B) glucose consumption, (C) L-threonine titer, and (D) L-threonine yield and specific L-threonine titer calculated from the data at 24 h. The data with different asterisks represent significant differences ($P < 0.05$), and those without asterisk represent no significant difference.

measured NADPH concentrations in cells cultivated with and without betaine additions during the fermentation processes (Table 1). The NADPH concentrations with betaine addition were always higher than those without betaine addition; in particular, at 18 h, the gap reached 21%. The enhanced production of NADPH by betaine supplementation was most likely because of the increased expression of *zwf*. We also determined the transcriptional levels of *gnd* and *icd* that are involved in NADPH synthesis, and found no obvious variations in response to betaine addition (Table S2).

The generation of NADPH is often a limiting step for optimizing the production of many industrially important chemicals, including amino acids. Over the past few decades, different strategies have been attempted to enhance the NADPH supply to improve valine [17], isoleucine [18], and lysine production [19], among which overexpressing *zwf* was a useful approach [20]. Compared with these genetic engineering methods, the herein employed betaine supplementation approach is quite simple and controllable.

3.4. Effects of zwf deletion on NADPH synthesis, bacterial growth, and threonine production

To confirm the effects of *zwf* expression on increased NADPH formation and threonine production, we constructed a *zwf* deletion strain THRDΔ*zwf*. This strain was also used to perform a 5 L fed-batch fermentation. To our surprise, there was no threonine that could be detected in the fermentation broth. The NADPH concentrations of THRDΔ*zwf* with and without betaine supplementations are presented in Table 1. Expectedly, much lower NADPH was synthesized in the *zwf* deletion strain than that of THRD, and comparable amounts of NADPH were produced at both conditions, either with or without betaine addition. The bacterial growth was promoted significantly by *zwf* knockout, and the final biomass increased by 25.6%. Like in the case for THRD, betaine addition also enhanced the growth of THRDΔ*zwf* (Fig. 1, Fig. 3). Collectively, the deletion of *zwf* extremely decreased NADPH synthesis and completely abolished the production of threonine, while significantly improved the bacterial growth. It is likely that threonine synthesis is hampered because of a lack of NADPH supply and a plenty of carbon flows into the anabolic reactions to build up the bacterial biomass. However, the detailed mechanism is unclear.

3.5. Stimulation of plasmid-based eGFP expression by betaine

Despite a significant increase in *zwf* expression in cells cultured with betaine addition, it is not convincing to conclude that there is a strong connection between *zwf* expression and betaine. Therefore, we constructed a plasmid in which the *zwf* promoter was installed to activate eGFP expression and transfer it into competent *E. coli* THRD and DH5α cells. The shake-flask culture of THRD was used to simulate

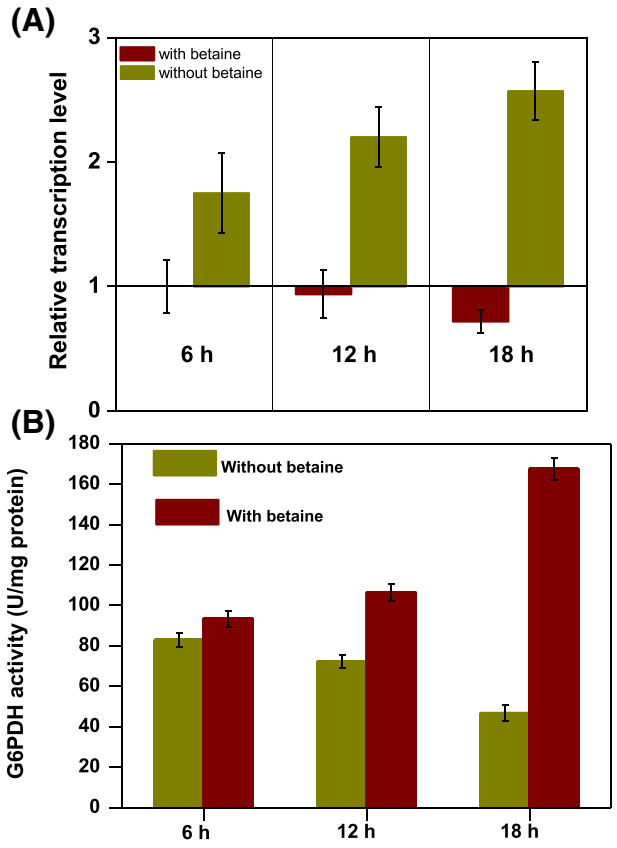

Fig. 2. Relative transcript levels of *zwf* in *Escherichia coli* THRD cultured with betaine or without betaine addition (A) and enzyme activities of glucose-6-phosphate dehydrogenase with or without betaine addition (B). The relative mRNA levels were compared using reverse transcription quantitative polymerase chain reaction and the mRNA level at 6 h without betaine addition was set to 1.

L-threonine fermentation with the same betaine-adding procedure as in the fermenter. The fluorescence intensity detection results showed a higher eGFP expression when the betaine was supplemented (Fig. 4A). Meanwhile, DH5α was cultured in LB media in flasks supplemented with different concentrations of betaine up to 1 g/L, beyond which the cell growth would be severely hindered. The fluorescence intensity per unit OD_{600} was perfectly correlated to the amount of betaine added during the cultivation process (Fig. 4B). To rule out the possibility that the higher fluorescence intensity was detected because of the increased cell membrane permeability (or other reasons) with betaine addition, another plasmid was constructed in which eGFP was expressed under the *gapA* promoter that encodes glyceraldehyde 3-phosphate dehydrogenase, and we found no correlation between fluorescence intensity and betaine addition (Fig. S2). Together, these results sufficiently proved a transcriptional-level response of *zwf* as a result of the betaine addition in *E. coli* strains.

Table 1
NADPH levels of *Escherichia coli* THRD and THRDΔ*zwf* cultured with or without betaine supplementation.

Strain	Betaine	NADPH (nmol/g WCW[a])		
		6 h	12 h	18 h
THRD	Without betaine	249.4 ± 1.8	239.0 ± 2.1	218.1 ± 3.0
	With betaine	289.9 ± 2.7	272.4 ± 2.1	263.9 ± 2.6
THRDΔ*zwf*	Without betaine	98.9 ± 1.6	117.4 ± 2.2	139.2 ± 2.7
	With betaine	96.8 ± 1.5	115.6 ± 2.3	140.3 ± 1.8

[a] WCW, wet cell weight.

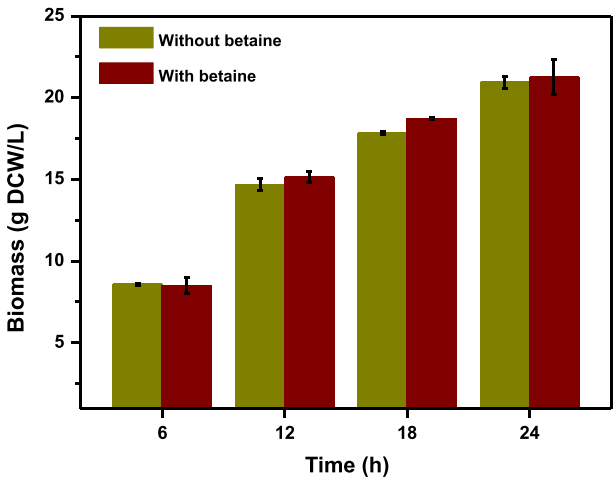

Fig. 3. Biomass of *Escherichia coli* THRDΔ*zwf* cultured in a 5 L fermenter with or without betaine supplementation.

The nucleotide sequences of *zwf* promoter are shown in Fig. S3A; the minimal region with -35 and -10 hexamers ensures its transcription, while the sequences upstream -35 box are involved in regulations by

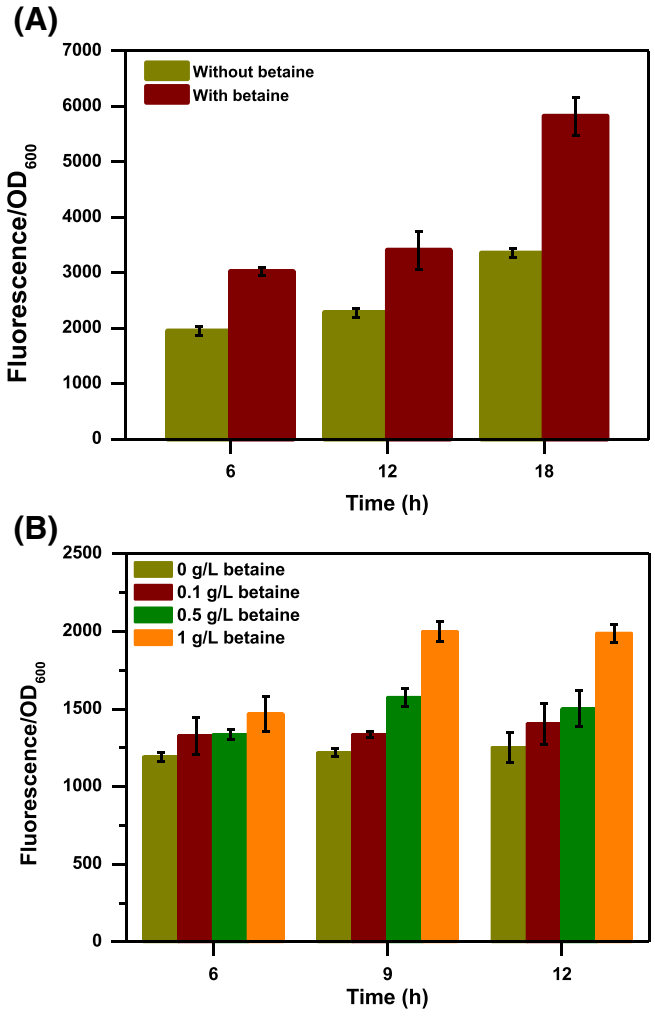

Fig. 4. Changes of fluorescence intensity emitted by *Escherichia coli* THRD/pUC-P_{zwf}-*egfp* during L-threonine shake-flask fermentation with and without betaine additions (A) and profiles of fluorescence intensity emitted by *E. coli* DH5α cultured in LB media supplemented with different concentrations of betaine (B).

Table 2
Comparison of fermentation parameters of *Escherichia coli* THPZ at 24 h in fed-batch cultures supplemented with or without betaine.

Betaine	Biomass (g DCW/L)	Glucose consumption (g/L)	L-Threonine titer (g/L)	L-Threonine productivity (g/L/h)	L-Threonine yield (g/g glucose)	Specific L-threonine titer (g/g DCW[a])
Without	17.6 ± 0.3	226.8 ± 9.9	107.3 ± 1.9	4.47 ± 0.08	0.47 ± 0.01	6.11 ± 0.05
With	18.7 ± 1.5	235.1 ± 15.6	126.1 ± 3.0	5.26 ± 0.12	0.54 ± 0.02	6.74 ± 0.30

[a] DCW, dry cell weight.

transcript factors [21,22,23]. Although betaine, as a signal molecule, might directly bind to the *zwf* promoter region, the regulation of *zwf* expression by betaine is mostly probably indirect. Niazi et al. [24] reported that the *zwf* promoter is induced by superoxide-generating agents, such as ethyl viologen and benzyl viologen, and alkylating agents. Some type of transcription factor is likely to be involved in this regulation, e.g., *zwf* transcription was reportedly activated by SoxS, MarA, and Robs in *E. coli* [23]. Global transcription factor(s) might also participate in the regulation of *zwf* expression considering that the catabolite repressor activator (*Cra*) was linked to expression of the osmotically induced operon *betAB*, whose products convert choline into betaine [25]. Apart from the regulation by a transcription factor, the possibility of *zwf* transcriptionally or translationally regulated by a riboswitch that senses betaine cannot be ruled out. Fig. S3B shows the predicted secondary structure of 5′-UTR of *zwf* mRNA [21]. In nature, riboswitches such as the lysine riboswitch are widely distributed in the bacterial domain [26].

3.6. Promotion of L-threonine fermentation by substituting ppc promoter with zwf promoter

To demonstrate the application potential of this novel finding, we substituted the promoter of *ppc* in THRD with P*zwf* and named the newly constructed strain as THPZ. The *ppc* gene encodes PEP carboxylase, which catalyzes PEP carboxylation reaction to supply oxaloacetate, the precursor for L-threonine synthesis. Therefore, high-level *ppc* expression was critical for L-threonine production. This anaplerotic reaction would not only benefit bacterial growth because the TCA cycle was enhanced but also improve L-threonine yield due to the CO_2-fixing property. The comparative fermentation parameters with and without betaine additions are shown in Table 2. The biomass, L-threonine titer, and yield of THPZ cultured without betaine addition were slightly higher than those of THRD. This finding suggested that P*zwf* of *E. coli* might be transcriptionally stronger than P*ppc*. Even so, with betaine supplementation, the L-threonine titer of THPZ increased further by 17.6% (Table 2), indicating an iterative effect of the betaine addition, besides the promotive effects of the promoter exchange. However, it is impossible to distinguish this iterative effect from the result of the enhanced NADPH or oxaloacetate supply although both may play a role.

4. Conclusions

In summary, we revealed a novel mode of betaine facilitating the microbial production of useful compounds. Increases in *zwf* transcription and G6PDH activity followed by higher NADPH pools in the presence of betaine may be beneficial for the cell growth, and thus promote the production of L-threonine. The enhanced expression of *zwf* resulting from the betaine addition is of great importance for the production of NADPH-dependent aspartate-family amino acids (such as L-threonine, L-lysine, and L-isoleucine) and derivatives as well as aromatic amino acids whose syntheses demand a supply of the precursor erythrose 4-phosphate in the PPP. On the other hand, the *zwf* promoter is useful when placed before the key genes for synthesizing valuable chemicals that can be induced to express by the addition of betaine at certain time points as required. With increased understanding of the action mechanisms of betaine, we anticipate an increase in its applications in the microbial fermentation industry. In addition, the good biocompatibility, reasonable carbon-to-nitrogen ratio, low cost, and low supplementation amount make it feasible to add betaine to industrial fermentation media.

Competing interests

The authors declare no conflict of interest.

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Supplementary material

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