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BIOENERGY AND BIOFUELS

Improving the fermentation performance of *Clostridium acetobutylicum* ATCC 824 by strengthening the VB1 biosynthesis pathway

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Abstract

Vitamin B1 (VB1) is an essential coenzyme for carbohydrate metabolism and involved in energy generation in most organisms. In this study, we found that insufficient biosynthesis of VB1 in *Clostridium acetobutylicum* ATCC 824 is a major limiting factor for efficient acetone-butanol-ethanol (ABE) fermentation. In order to improve the fermentation performance of *C. acetobutylicum* ATCC 824, the VB1 biosynthesis pathway was strengthened by overexpressing the *thiC*, *thiG*, and *thiE* genes. The engineered strain 824(thiCGE) showed enhanced VB1 and energy synthesis, resulting in better growth, faster sugar consumption, higher solvents production, and lower acids formation than the wild-type strain in both VB1 free and normal P2 medium (1 mg/L). Compared with the wild-type strain, 824(thiCGE) produced 13.0 ± 0.1% or 12.7 ± 1.2% more butanol in VB1 free P2 medium when glucose or xylose was used as the substrate, respectively. When mixed sugar (glucose:xylose = 2:1) was used as the substrate in VB1 free P2 medium, the xylose consumption rate and butanol titer of 824(thiCGE) were 45.8 ± 1.9% and 20.4 ± 0.3% higher than those of the wild-type strain. All these results demonstrated that this metabolic engineering strategy could provide a new and effective way to improve the cellular performance of solventogenic clostridia. In addition, it may have some potential application value in ABE fermentation using simple medium and/or lignocellulosic biomass.

Keywords *Clostridium acetobutylicum* · Vitamin B1 biosynthesis · Carbohydrate metabolism · Acetone-butanol-ethanol fermentation

Introduction

Clostridium acetobutylicum is an attractive anaerobe that produces the solvents acetone, butanol, and ethanol (ABE) in a ratio of 3:6:1 (Lütke-Eversloh and Bahl 2011). Among them, butanol is the most important product due to its higher production and economic values. In addition to be an important chemical precursor for paints, polymers and plastics, butanol is also considered as an ideal biofuel for replacing fossil fuels (Lee et al. 2008). Although it has broad application prospects, low product titer and productivity due to the inefficient fermentation process led to poor economic benefit, which has seriously hindered the industrialization of biobutanol. In order to overcome these obstacles, various strategies have been developed, such as strain screening (Formanek et al. 1997; Guo et al. 2011), adaptive engineering (Isar and Rangaswamy 2012; Yang and Zhao 2013), genetic modification (Harris et al. 2001; Harris et al. 2000; Jang et al. 2012; Nair et al. 1999; Zhu et al. 2011), and process optimization (Ezeji et al. 2004a; Ezeji et al. 2004b; Groot' et al. 1984; Xue et al. 2012). Recently, some efforts have been undertaken to improve the fermentation performance of *C. acetobutylicum*, which focused on cell growth factor, such as flavonoids (Wang et al. 2014) and biotin (Yang et al. 2016).

Vitamin B1 (VB1), namely thiamine, is an essential cell growth factor required by most organisms. It is very crucial for a number of central metabolic processes, such as glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle

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(Bazurto et al. 2015). In these biochemical reactions, thiamin is served as a cofactor in thiamin pyrophosphate-dependent enzymes, including pyruvate dehydrogenase, transketolase, α-ketoglutarate dehydrogenase, pyruvate decarboxylase, branched-chain α-ketoacid dehydrogenase, and so on (Frank et al. 2007), most of which are relevant to ATP or NAD(P)H. Putative genes responsible for VB1 biosynthesis in *C. acetobutylicum* ATCC 824 were identified (Fig. 1), indicating that VB1 might be produced during the fermentation. However, it still needs to add a dose of VB1 to the fermentation medium, such as P2 medium (Baer et al. 1987), to maintain normal cell physiological and efficient ABE production.

VB1 is composed of a pyrimidine ring and a thiazole ring, and these two rings were linked by a methylene bridge (Manzetti et al. 2014). In prokaryotes (e.g., *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium*), there are many enzymes involved in VB1 biosynthesis (Begley et al. 1999). For example, ThiF, ThiS, ThiG, ThiH, and ThiI are participated in the thiazole ring biosynthesis, ThiC is required for the pyrimidine ring biosynthesis, and ThiE is necessary for the catalytic reaction that linking of the thiazole ring and pyrimidine ring. In addition, some kinase also involved in VB1 biosynthesis, such as ThiL, PdxK, ThiD, and ThiM (Begley et al. 1999). In *C. acetobutylicum* ATCC 824, Dxs, NifS, ThiI, ThiH, ThiG, ThiC, ThiK, and ThiE may be responsible for VB1 biosynthesis based on the annotated in the genome of *C. acetobutylicum* ATCC 824 (Nolling et al. 2001) (Fig. 1).

In this study, in order to improve the fermentation performance of *C. acetobutylicum* ATCC 824, the VB1 biosynthesis pathway was strengthened by overexpressing three essential genes, including *thiC*. (encoding phosphomethylpyrimidine synthase), *thiG* (encoding thiazole synthase), and *thiE* (encoding thiamine monophosphate synthase) (Yu et al. 2015; Settembre et al. 2003). The results showed that when using glucose or xylose as the substrate, the fermentation performance of the engineered strain 824(thiCGE) was significantly improved. What is more, 824(thiCGE) also exhibited stronger mixed sugars utilizing ability. Therefore, this work revealed that VB1 is a pivotal cell growth factor for *C. acetobutylicum* ATCC 824, and the engineering strategy employed here could provide a new and effective way to improve the cellular performance of solventogenic clostridia.

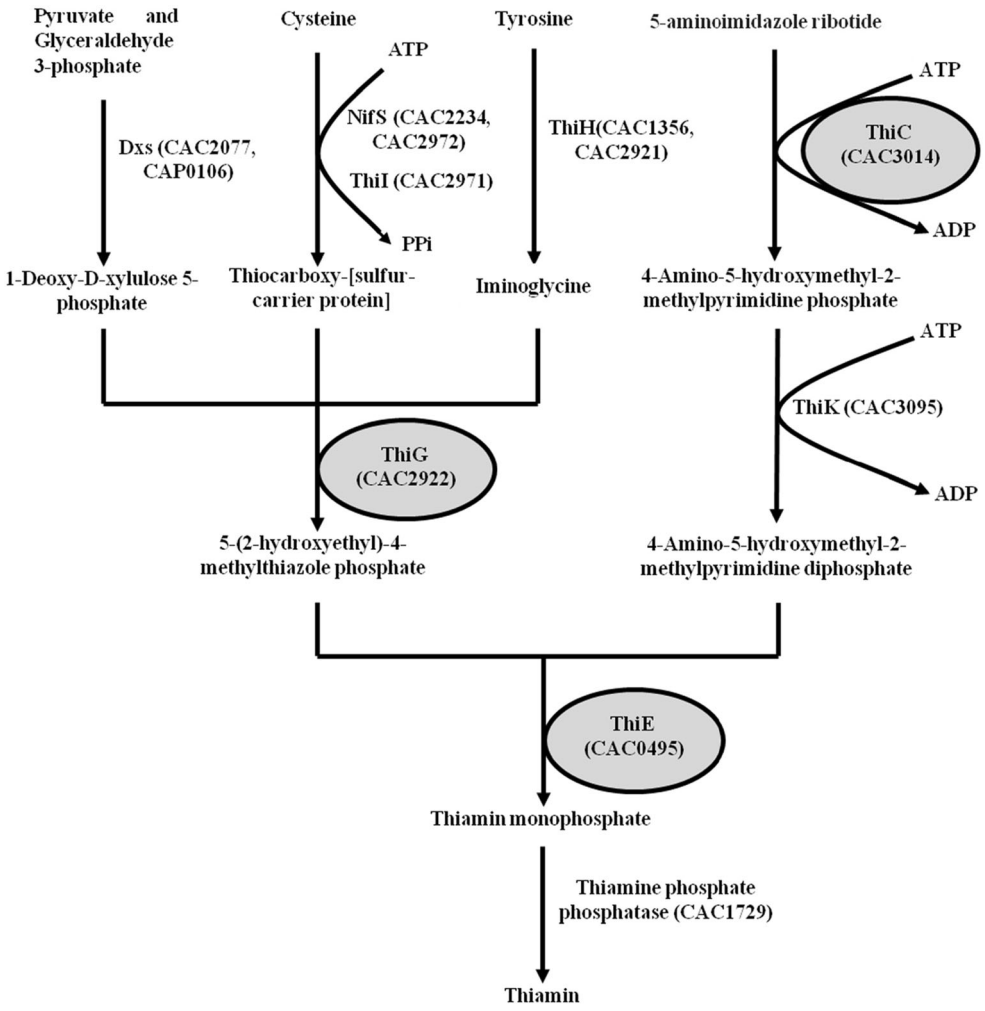

Fig. 1 The pathway of VB1 biosynthesis in *C. acetobutylicum* ATCC 824. The enzymes overexpressed in this study are showed in gray oval. ThiC, phosphomethylpyrimidine synthase; ThiG, thiazole synthase; ThiE, thiamine monophosphate synthase

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are showed in Table 1. The plasmids pMTL82151 (Heap et al. 2009) and pAN2 (Heap et al. 2007) and strain *C. acetobutylicum* ATCC 824 were supplied by Prof. Shang-Tian Yang from the Ohio State University. *E. coli* DH5α was purchased from Tiangen (Beijing, China) and used for amplification and recombinant plasmids construction. *E. coli* TOP10 (bearing the plasmid pAN2) was used for amplification and methylation of the recombinant plasmids.

Growth conditions

E. coli DH5α and *E. coli* TOP10 were grown aerobically at 37 °C in liquid Luria-Bertani (LB) medium or on LB agar, and 25 μg/mL chloramphenicol or 20 μg/mL tetracycline was

Table 1 Strains, plasmids, and primers used in this study

Strain/plasmid	Relevant characteristic	Source or reference
Strains		
C. acetobutylicum	ATCC 824	ATCC
E. coli DH5α	*DeoR, recA1, endA1, hsdR17*(rk^-, mk+)	TIANGEN
E. coli Top10	*hsd R*, *mcr A*, *rec A1*, *end A1*	TIANGEN
Plasmids		
pAN2	Φ3t I, p15A ori, Tet^R	(Heap et al. 2007)
pMTL82151	ColE1 ori; Cm^R; pBP1 ori; TarJ	(Heap et al. 2009)
pMTL-Pthl	From pMTL82151; P*thl*	This work
pMTL-Pthl thiCGE	From pMTL82151; P*thl*::*thiC* + *thiG* + *thiE*	This work
Primers for gene amplification	Sequence (5′-3′)	
thiC-F	AGGAGGTTAGTTAGAGGATCCTTAAAGGAGAGTATATTATG GATTACAC	
thiC-R	CCACTTAAATTCCTCCCAAAACCCTAACTTACTTAAAAATTAAT CCTCTC	
thiG-F	GAGAGGATTAATTTTTAAGTAAGTTAGGGTTTTGGGAGGAATTT AAGTGG-	
thiG-R	CACAAAAACTCCTCCTAACCTCCCTACACTTCCTATCTTAAAAA TCCTG-3′	
ThiE-F	AGGATTTTTAAGATAGGAAGTGTAGGTGAGGTTAGGAGGAGTTT TTGTG	
thiE-R	ACGACGGCCAGTGCCAAGCTTGTTTTGGTGTATACGACTTA TTTAGTTG	
thiGE-F	GAGAGGATTAATTTTTAAGTAAGTTAGGGTTTTGGGAGGAATTT AAGTGG	
thiGE-R	ACGACGGCCAGTGCCAAGCTTGTTTTGGTGTATACGACTTA TTTAGTTG	
thiCGE-F	AGGAGGTTAGTTAGAGGATCCTTAAAGGAGAGTATATTATG GATTACAC	
thiCGE-R	ACGACGGCCAGTGCCAAGCTTGTTTTGGTGTATACGACTTA TTTAGTTG	
pMTL-F	TGAAGTACATCACCGACGAGCAAG	
pMTL-R	TGCTGCAAGGCGATTAAGTTGGGT	
Primers for RT-PCR		
thiC-F	TGCAGGTTATGGTTGAAGGTCCAG	
thiC-R	CTAGCAGCAGCAACAGCTCCTC	
thiG-F	GAGCAGCAGCAGTTATGCCA	
thiG-R	AGTACCGCATCTGCTCCCAT	
ThiE-F	ATGCCGCTGATTAAGGCACG	
thiE-R	GCTTGAGCATCACCCTTGGT	
CAC2679-F	GACATTACTTCAAACGAACCTG	
CAC2679-R	CCCTTAGCCCATTTATTCCT	

added as needed. *C. acetobutylicum* ATCC 824 or its derived strains were grown anaerobically at 37 °C in liquid reinforced clostridial medium (RCM) (Ventura et al. 2013) or on RCM agar (supplemented with 30 μg/mL thiamphenicol as needed). P2 medium (pH 6.5) (Liao et al. 2017) (supplemented with 30 μg/mL thiamphenicol as needed) was used for batch fermentation.

DNA manipulation and transformation

Axygen®AxyPrep™ Genomic DNA Kit (Corning, Wujiang, China) was used for isolating the genomic DNA from *C. acetobutylicum* ATCC 824, and Axygen® AxyPrep™ Plasmid Kit (Corning, Wujiang, China) was used for isolating the plasmids DNA from *E. coli* strains. Restriction enzymes used in this study were purchased from Thermo Scientific (Shanghai, China). PCR primers were synthesized by Sangon Biotech (Shanghai, China) and DNA ploymerase were purchased from Takara Biomedical Technology (Beijing, China). Table 1 also lists the PCR primers used in this study. Firstly, the genes *thiC* (CAC3014), *thiG* (CAC2922), and *thiE* (CAC0495) involved in VB1 biosynthesis were PCR amplified from the genomic of *C. acetobutylicum* ATCC 824 using the corresponding primers. Subsequently, the *thiG* and *thiE* were ligated through overlap PCR using *thiG* and *thiE* fragments as the templates and *thiGE*-F/*thiGE*-R as the primers, yielding gene fragment of *thiGE*. Finally, the gene fragment of *thiC* and *thiGE* were ligated through overlap PCR using *thiC* and *thiGE* fragments as the templates and *thiCGE*-F/*thiCGE*-R as the primers, yielding gene fragment of *thiCGE*. Then, the *thiCGE* fragment was cloned into the plasmid pMTL-Pthl, which was digested with *Bam*HI and *Hin*dIII using ClonExpress II One Step Cloning Kit (Vazyme Biotech Co. ,Ltd., Nanjing, China), yielding the recombinant plasmid pMTL-Pthl thiCGE. The recombinant plasmid was first transferred into *E. coli* TOP10 bearing the plasmid pAN2 for methylation. Then, the methylated recombinant plasmid was isolated and transferred into *C. acetobutylicum* ATCC 824 by electrotransformation as described previously (Liao et al. 2017). The primers pMTL-F/pMTL-R (Table 1) were used to identify the recombinant strains.

RNA isolation and reverse transcription-PCR analysis

C. acetobutylicum strains were inoculated in serum bottles with VB1 free P2 medium containing 80 g/L glucose and grown anaerobically at 37 °C. The samples were taken at 12, 24, and 36 h, and the cell was collected by centrifugation. The total RNA was isolated using RNAprep pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China). The PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology, Dalian, China) was used for cDNA Synthesis, and SYBR Premix Ex Taq II (2×) (Tli RNaseH Plus), Bulk (Takara Biomedical Technology, Beijing, China) was used for real-time PCR. PCR reactions were carried out in a LightCycler®96 instrument (Roche, Switzerland) with the protocol: 30 s at 95 °C, then 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. Each transcript was performed with three PCR reactions. The pullulanase gene (CAC2679) was used as housekeeping genes (Tomas et al. 2003; Tseng et al. 2001). Primers for housekeeping genes and VB1 biosynthesis gene (*thiC*, *thiG*, and *thiE*) are listed in Table 1.

VB1 and ATP assays

The wild-type and 824(thiCGE) strains were inoculated in serum bottles with VB1 free P2 medium containing 80 g/L glucose and grown anaerobically at 37 °C. The samples were taken at various time points. The supernatant was collected by centrifugation and used for VB1 assay, while the cell pellets were resuspended in lysate. After the cell pellets were completely lysed, the supernatant was collected by centrifugation and used for ATP assay. The Vitamin B1 Assay Kit (Cablebridge Biotechnology, Shanghai, China) and ATP Assay Kit (Beyotime, Shanghai, China) were used for VB1 and ATP assay, respectively, according to the recommendation of the manufacturer.

Fermentation studies

C. acetobutylicum ATCC 824 or the engineered strain 824(thiCGE) was precultured to the early stationary phase in liquid RCM (supplemented with 30 μg/mL thiamphenicol as needed) as the seed culture. P2 medium with various concentrations of VB1 (0, 1, 3, or 5 mg/L) was used for ABE fermentation, and 1 mg/L represents the dose commonly added in normal P2 medium. Batch fermentations were performed in 50 mL P2 medium in serum bottles with glucose (80 g/L), xylose (80 g/L), or mixed sugars (40 g/L glucose and 20 g/L xylose). The serum bottles were filled with nitrogen to establish anaerobic conditions. The samples were taken at regular intervals to monitor the cell growth, sugar consumption, and products formation.

Analytical methods

High-performance liquid chromatography (HPLC; Waters 2695, Milford, MA) was used for measuring the concentration of sugar (Liao et al. 2017). And the concentration of products in the fermentation broth were determined by gas chromatograph (Agilent 7890A GC, Agilent Technologies) as described previously (Liao et al. 2017).

Results

Effect of exogenous VB1 addition on the fermentation performance of *C. acetobutylicum*

The P2 mediums with different concentrations (0, 1, 3, or 5 mg/L) of VB1 were used to study the effects of VB1 on the fermentation performance of *C. acetobutylicum* ATCC 824. The cell growth, glucose consumption and products formation were examined, and the results are shown in Fig. 2. Fermentation without VB1 addition was used as the control. As the VB1 concentration increased from 0 to 3 mg/L, the cellular performance of *C. acetobutylicum* ATCC 824 was enhanced. The best results were obtained with 3 mg/L VB1, and the max OD_{600}, glucose consumption and total solvents titer were 7.5 ± 0.3, 79.01 ± 0.14 g/L, and 20.53 ± 0.27 g/L, respectively, which were significantly higher than those of the control (max OD_{600} = 6.0 ± 0.4, glucose consumption = 65.10 ± 0.57 g/L, total solvents titer = 15.90 ± 0.82 g/L). In addition from Fig. 2b, c, we can see that the glucose consumption rate and solvents formation rate were faster than the control, resulting in an increase solvents productivity. With 3 mg/L VB1, the residual glucose was only about 4.72 ± 1.35 g/L at 48 h, while it still had 20.43 ± 2.83 g/L for the control; the total solvents titer reached 20.18 ± 0.52 g/L at 48 h, which was much higher than that of the control (12.18 ± 0.49 g/L). These results indicated that VB1 plays an important role in cellular performance of *C. acetobutylicum* ATCC 824.

Real-time PCR and relative gene expression analysis

In general, transcriptions of *thiC*, *thiG*, and *thiE* in *C. acetobutylicum* ATCC 824 and 824(thiCGE) were increased from 12 to 24 h, then decreased at 36 h (Fig. 3). After the recombinant plasmid pMTL-Pthl thiCGE was transferred into *C. acetobutylicum* ATCC 824, all three measured genes were highly overexpressed. For example, the relative expression levels of *thiC*, *thiG*, and *thiE* of 824(thiCGE) were increased by 38.9 ± 4.6, 16.1 ± 2.7, and 41.4 ± 3.4-fold compared to those in the wild-type strain at 12 h (Fig. 3). The enhancement of the transcription level in 824(thiCGE) indicates that the *thiC*, *thiG*, and *thiE* were effectively overexpressed.

Effect of *thiC*, *thiG*, and *thiE* overexpressing on VB1 and ATP synthesis

As shown in Fig. 4a, the extracellular VB1 concentration was significantly increased during the logarithmic and stationary phases (0–36 h), then it was decreased at the end of the fermentation. During the whole fermentation process, the VB1 concentration of the engineered strain 824(thiCGE) was significantly higher than that of the wild-type strain. For example, VB1 produced by *C. acetobutylicum* was increased by 1.88 ± 0.09 times at 36 h, from 0.73 ± 0.07 to 2.10 ± 0.14 μM (~ 0.6 mg/L). It means that the metabolic engineering strategy to improve VB1 biosynthesis was succeeded by overexpressing *thiC*, *thiG*, and *thiE*.

Since VB1 is served as a coenzyme participating in some enzymatic catalytic processes of energy generation, such as glycolysis and tricarboxylic acid cycle (Manzetti et al. 2014), the ATP concentration was also measured during the fermentation process. As shown in Fig. 4b, the ATP concentrations of the engineered strain 824(thiCGE) and the wild-type strain were decreased gradually after 24 h; however, the ATP concentration of 824(thiCGE) was always higher than that of the wild-type strain. This result indicated that the energy generation was also enhanced by reinforcing the VB1 biosynthesis.

ABE fermentation using glucose as the substrate

In order to confirm the effect of VB1 biosynthesis on the fermentation performance of *C. acetobutylicum* ATCC 824, batch fermentation of glucose was carried out. The results showed that 824(thiCGE) exhibited better fermentation performance in both VB1 free and normal P2 medium than the

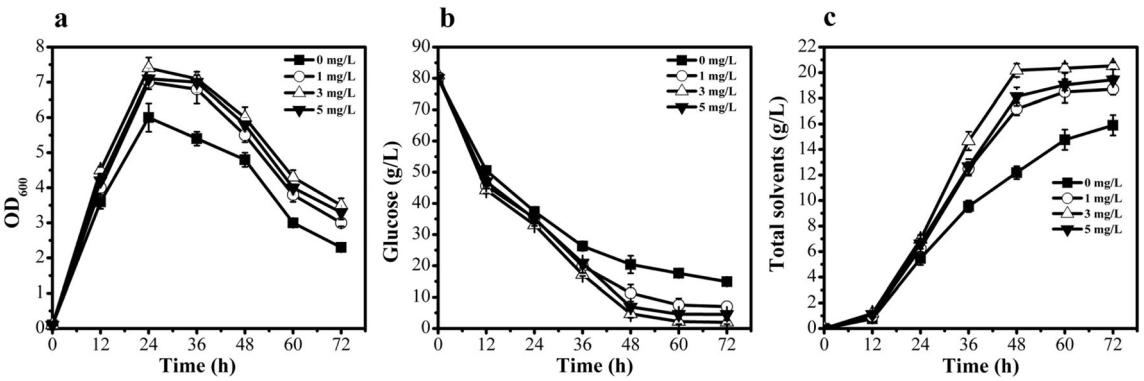

Fig. 2 Growth (**a**), glucose utilization (**b**), and solvent production (**c**) of *C. acetobutylicum* ATCC 824 fermentation with various concentrations of VB1. The data are the means and standard deviations of three replicates

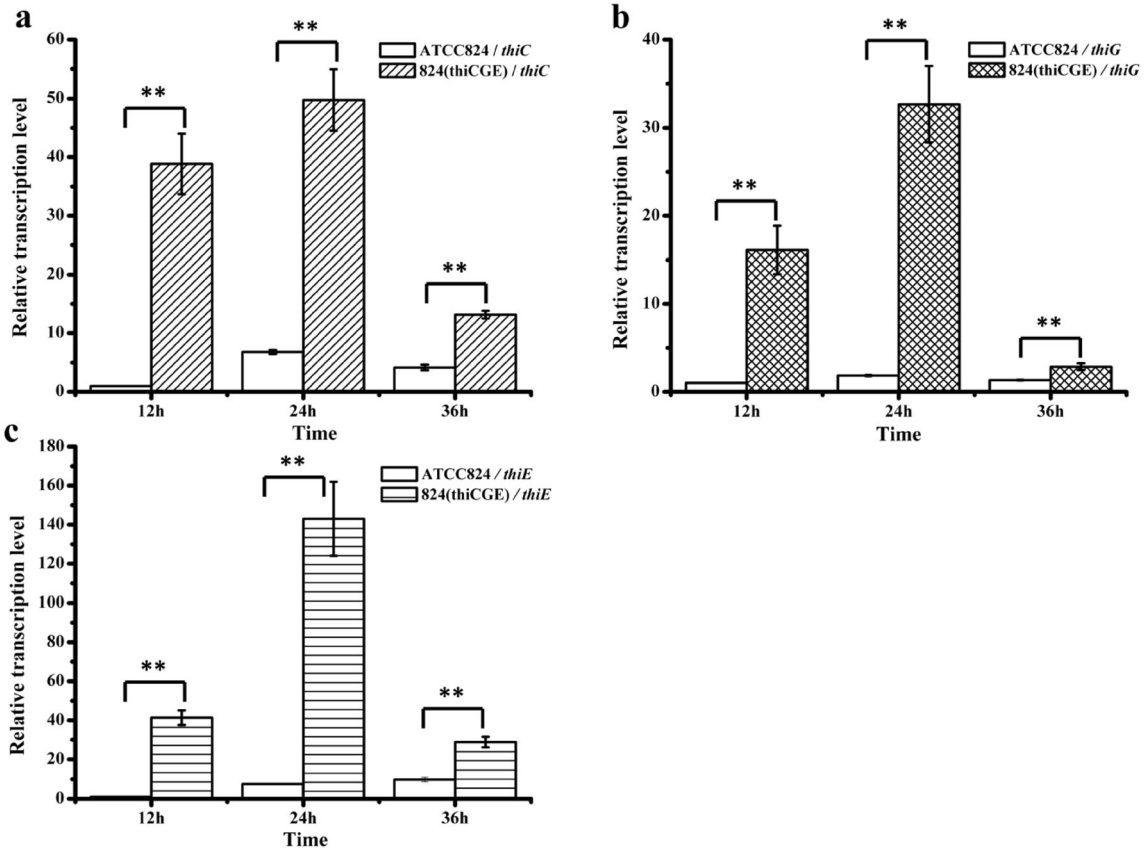

Fig. 3 Real-time PCR analysis of **a** *thiC*, **b** *thiG*, and **c** *thiE* transcripts in VB1 free P2 medium (80 g/L glucose) for *C. acetobutylicum* ATCC 824 and 824(thiCGE). The data are the means and standard deviations of three replicates (**$P \le 0.01$; $0.01 \le$ *$P \le 0.05$; *t* test)

wild-type strain (Table 2). When engineered strain 824(thiCGE) was cultured in the VB1 free P2 medium, the max OD_{600} reached up to 6.8 ± 0.2, which was significantly higher than that of the wild-type strain (max OD_{600} = 5.4 ± 0.3) (Fig. 5a). The engineered strain 824(thiCGE) also showed much faster glucose consumption rate and solvents production rate (Fig. 5a, b). As a result, butanol productivity was increased by 12.6 ± 0.1% compared with the wild-type strain. The total solvents produced by 824(thiCGE) reached 18.61 ± 0.22 g/L, 16.8 ± 0.9% higher than that of the wild-type strain (Fig. 5b). In addition, the organic acids concentrations of 824(thiCGE) were always lower than those of the wild-type strain throughout the fermentation process (Fig. 5c). Similar phenomenon was observed when *C. acetobutylicum* was cultured in the normal P2 medium (Fig. 5d–f). It should be noted that the fermentation performance of 824(thiCGE) in VB1 free P2 medium was comparable to the wild-type strain in normal P2 medium, indicating that the enhanced VB1 biosynthesis in 824(thiCGE) could replace exogenous VB1 addition in normal P2 medium (Table 2).

ABE fermentation using xylose or mixed sugars as the substrate

Since VB1 also plays an important role in the pentose phosphate pathway (Bazurto et al. 2015; Manzetti et al.

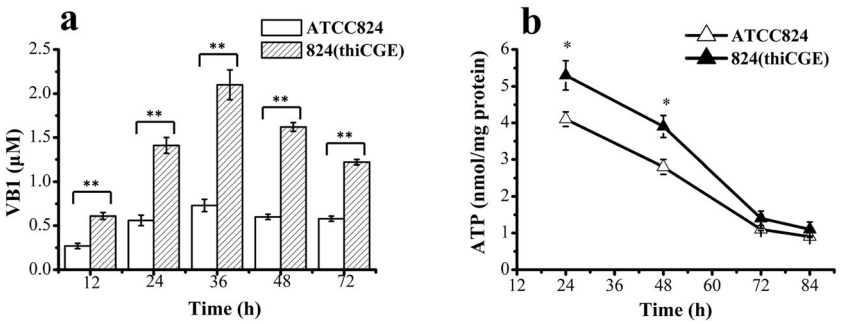

Fig. 4 VB1 (**a**) and ATP (**b**) production of *C. acetobutylicum* ATCC 824 and 824(thiCGE) during the whole batch fermentation process in VB1 free P2 medium (80 g/L glucose). The data are the means and standard deviations of three replicates (**$P \le 0.01$; $0.01 \le$ *$P \le 0.05$; *t* test)

Table 2 Batch fermentation of *C. acetobutylicum* ATCC 824 and 824(thiCGE) using VB1 free or normal P2 medium with 80 g/L glucose in serum bottles

	VB1	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Butanol yield (g/g)	Butanol productivity (g/L/h)	Total solvent (g/L)	Max OD_{600}	Specific growth rate (h^{-1})	Total sugar consumption (g/L)
ATCC 824	–	5.51 ± 0.18	9.68 ± 0.06	0.82 ± 0.13	2.40 ± 0.28	1.72 ± 0.28	0.155 ± 0.001	0.135 ± 0.001	15.94 ± 0.31	5.4 ± 0.3	0.166 ± 0.001	62.52 ± 0.33
ATCC 824	+	6.11 ± 0.16	11.00 ± 0.14	1.62 ± 0.28	1.28 ± 0.07	0.72 ± 0.14	0.156 ± 0.004	0.152 ± 0.004	18.68 ± 0.42	7.4 ± 0.2	0.179 ± 0.001	70.51 ± 2.23
824(thiCGE)	–	5.95 ± 0.09	10.94 ± 0.08	1.67 ± 0.08	1.51 ± 0.06	0.98 ± 0.21	0.156 ± 0.004	0.152 ± 0.001	18.61 ± 0.22	6.8 ± 0.2	0.176 ± 0.001	69.74 ± 2.08
824(thiCGE)	+	7.21 ± 0.18	13.18 ± 0.23	2.03 ± 0.13	0.80 ± 0.06	0.40 ± 0.03	0.164 ± 0.002	0.183 ± 0.003	22.44 ± 0.66	7.3 ± 0.2	0.179 ± 0.001	80.13 ± 0.93

"±" average of three replicates, "–" represent did not add VB1, "+" represent added 1 mg/L VB1

2014; Tittmann 2009), then we assumed that the enhanced VB1 biosynthesis could also promote the xylose metabolism. In order to confirm this hypothesis, the ABE fermentation by *C. acetobutylicum* ATCC 824 using 80 g/L xylose as the substrate was carried out. From Fig. 6, we can see that the xylose consumption rate and solvents production rate of *C. acetobutylicum* in normal P2 medium (Fig. 6d–f) were significantly faster than that in VB1 free P2 medium (Fig. 6a–c). The final butanol titer and xylose consumption of *C. acetobutylicum* ATCC 824 in normal P2 medium were 10.40 ± 0.11 g/L and 66.32 ± 0.18 g/L, respectively, 16.1 ± 2.3% and 14.1 ± 0.1% higher than those in VB1 free P2 medium (Table 3). As expected, compared with the wild-type strain, the engineered strain 824(thiCGE) exhibited better fermentation performances in both VB1 free and normal P2 medium (Fig. 6 and Table 3), although xylose utilization was much slower than glucose. When xylose fermentation was carried out in VB1 free and normal P2 medium, the butanol titer of 824(thiCGE) were 10.10 ± 0.21 and 11.95 ± 0.21 g/L, respectively, which were 12.7 ± 1.2% and 14.9 ± 0.8% higher than those of the wild-type strain (Table 3).

In addition, the fermentation performance was also studied on mixed sugars containing 40 g/L glucose and 20 g/L xylose, based on the general ratio of glucose and xylose in the lignocellulosic hydrolysate (Aristidou and Penttilä 2000). The results showed that the engineered strain 824(thiCGE) exhibited better growth, faster sugars consumption, higher solvents production, and lower acids formation than those of the wild-type strain (Fig. 7 and Table 4), as observed in glucose or xylose fermentation.

Discussion

Improving the fermentation performance is very important for ABE fermentation. Previous studies have shown that VB1 is a crucial molecule in some enzymatic catalytic processes of carbohydrate metabolism, which participate in energy generation from different carbohydrate sources. These enzymatic catalytic processes involves in glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle (Bazurto et al. 2015; Manzetti et al. 2014). In addition, VB1 could improve the cellular resistance to oxidative stress, heat stress, high light stress, and biotic stresses (Dong et al. 2015; Medina-Silva et al. 2006; Rapala-Kozik et al. 2008; Tunc-Ozdemir et al. 2009; van der Graaff et al. 2004; Wolak et al. 2015). In this study, we found that the cellular performance of *C. acetobutylicum* ATCC 824 was greatly affected by VB1. Although the putative genes responsible for VB1 biosynthesis were found in *C. acetobutylicum* ATCC 824 (Fig. 1), the VB1 biosynthesis was insufficient to maintain normal cell

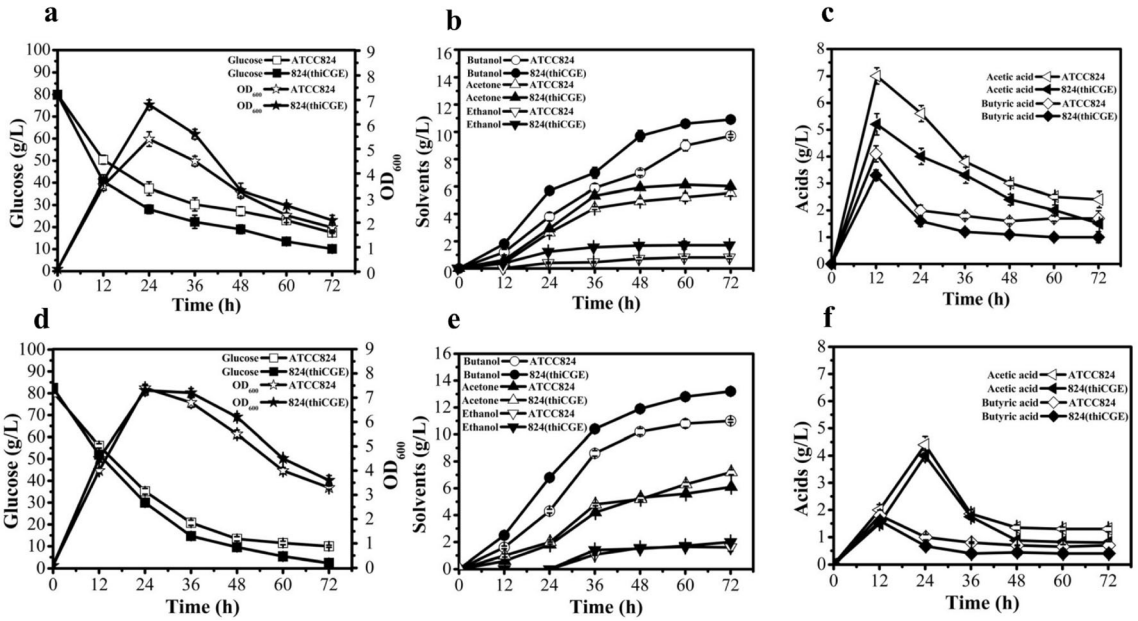

Fig. 5 Batch fermentation profiles of *C. acetobutylicum* ATCC 824 and 824(thiCGE) cultivated in VB1 free P2 medium and in normal P2 medium with 80 g/L glucose. **a** The profiles of growth and glucose consumption in VB1 free P2 medium. **b** The profiles of solvents production in VB1 free P2 medium. **c** The profiles of organic acids production in VB1 free P2 medium. **d** The profiles of growth and glucose consumption in normal P2 medium. **e** The profiles of solvents production in normal P2 medium. **f** The profiles of organic acids production in normal P2 medium. The data are the means and standard deviations of three replicates

growth and efficient solvent production (Fig. 2). With the VB1 concentration increasing, *C. acetobutylicum* ATCC 824 exhibited better growth, faster sugar utilization, and solvent production, indicating that VB1 play a key role for efficient ABE fermentation.

In order to improve the fermentation performance of *C. acetobutylicum* ATCC 824, the VB1 biosynthesis pathway was strengthened by overexpressing three essential genes, including *thiC*, *thiG*, and *thiE* (Fig. 1). Although there are many enzymes participating in VB1 biosynthesis, and

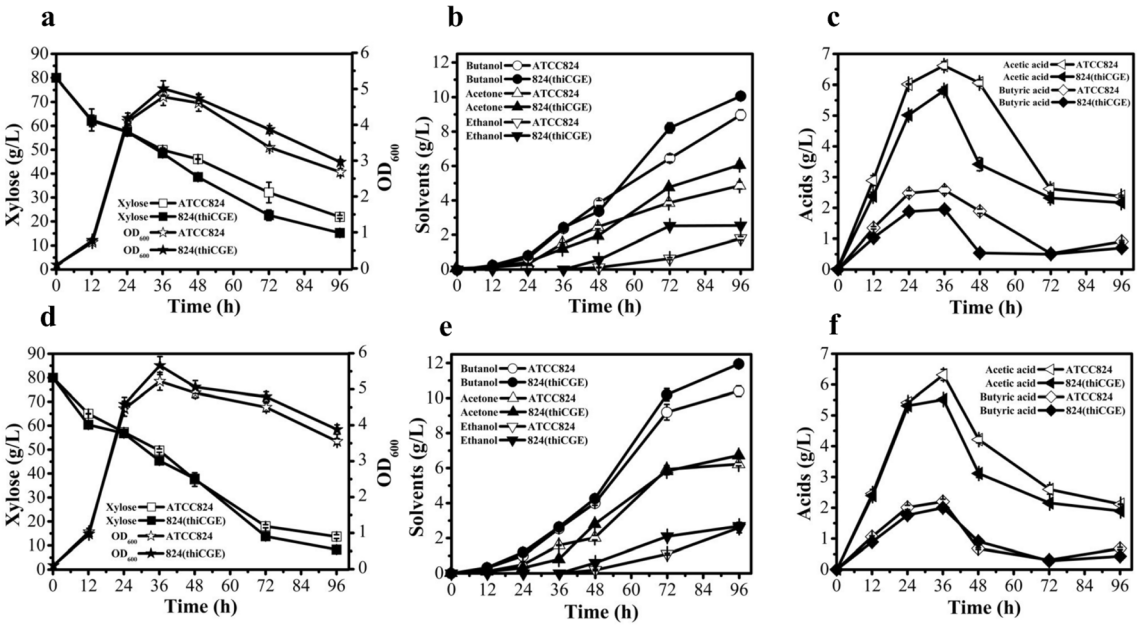

Fig. 6 Batch fermentation profiles of *C. acetobutylicum* ATCC 824 and 824(thiCGE) cultivated in VB1 free P2 medium and in normal P2 medium with 80 g/L xylose. **a** The profiles of growth and xylose consumption in VB1 free P2 medium. **b** The profiles of solvents production in VB1 free P2 medium. **c** The profiles of organic acids production in VB1 free P2 medium. **d** The profiles of growth and xylose consumption in normal P2 medium. **e** The profiles of solvents production in normal P2 medium. **f** The profiles of organic acids production in normal P2 medium. The data are the means and standard deviations of three replicates

Table 3 Batch fermentation of *C. acetobutylicum* ATCC 824 and 824(thiCGE) using VB1 free or normal P2 medium with 80 g/L xylose in serum bottles

	VB1	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Butanol yield (g/g)	Butanol productivity (g/L/h)	Total solvent (g/L)	Max OD_{600}	Specific growth rate (h^{-1})	Total sugar consumption (g/L)
ATCC 824	–	4.82 ± 0.20	8.96 ± 0.28	1.80 ± 0.11	2.36 ± 0.03	0.91 ± 0.03	0.155 ± 0.001	0.093 ± 0.001	15.58 ± 0.34	4.8 ± 0.2	0.111 ± 0.001	58.10 ± 0.23
ATCC 824	+	6.22 ± 0.11	10.40 ± 0.11	2.60 ± 0.25	2.10 ± 0.01	0.68 ± 0.04	0.157 ± 0.001	0.108 ± 0.004	19.27 ± 0.44	5.2 ± 0.3	0.111 ± 0.000	66.32 ± 0.18
824(thiCGE)	–	6.03 ± 0.21	10.10 ± 0.21	2.48 ± 0.06	2.15 ± 0.07	0.70 ± 0.06	0.156 ± 0.002	0.105 ± 0.002	18.58 ± 0.1	5.0 ± 0.2	0.112 ± 0.001	64.84 ± 0.49
824(thiCGE)	+	6.73 ± 0.15	11.95 ± 0.21	2.69 ± 0.11	1.88 ± 0.05	0.43 ± 0.05	0.167 ± 0.003	0.125 ± 0.003	21.37 ± 0.47	5.7 ± 0.3	0.114 ± 0.001	71.71 ± 0.78

"±" average of three replicates, "–" represent did not add VB1, "+" represent added 1 mg/L VB1

most of them have been validated in some prokaryotes, such as *E. coli*, *B. subtilis*, and *S. typhimurium* (Begley et al. 1999). We choose these three enzymes based on the structure of VB1 and previous reports. VB1 is formed from a pyrimidine ring and a thiazole ring, which are linked by a methylene bridge; thus, there are separate biosynthetic pathways for the pyrimidine moiety and thiazole moiety. As shown in Fig. 1, pyrimidine is formed from 5-aminoimidazole ribotide, and ThiC (encoded by *thiC*) is the key enzyme participated in pyrimidine ring synthesis; thiazole is formed from pyruvate, glyceraldehyde 3-phosphate, tyrosine and cysteine, and the oxidative condensation of these metabolic products (1-deoxy-D-xylulose-5-phosphate, thiocarboxy-[sulfur-carrier protein], and iminoglycine) into thiazole is catalyzed by ThiG (encoded by *thiG*); ThiE (encoded by *thiE*) is necessary for linking of the pyrimidine moiety and thiazole moiety to form thiamin monophosphate (Chakravortty et al. 2015; Settembre et al. 2003). The function of these genes have been validated in other bacteria, pyrimidine was needed when *thiC* was knocked out, deletion mutations of *thiG* resulted in a thiazole auxotrophy, and mutants of *thiE* resulted in thiamin insufficient (Backstrom et al. 1995; Dong et al. 2015; Kelleher et al. 1998; Begley et al. 1999; Webb et al. 1996; Zhang et al. 1997). A previous study has also shown that the increase in VB1 concentration was not obvious in *Arabidopsis thaliana* when overexpressing *thiC* or *thi1* (equivalent to *thiG* in *C. acetobutylicum*) singly, while it was dramatically improved by overexpressing *thiC* and *thi1* simultaneously (Dong et al. 2015). Therefore, we attempted to reinforce the VB1 biosynthesis by co-expressing these three essential genes (*thiC*, *thiG*, and *thiE*). The results showed that both the transcript levels of the *thiC*, *thiG*, and *thiE* (Fig. 3) and VB1 concentration (Fig. 4a) of the engineered strain 824(thiCGE) were significantly higher than those of the wild-type strain, demonstrating that this metabolic engineering strategy was successful in *C. acetobutylicum*.

The fermentation results in VB1 free P2 medium showed that both the total glucose consumption and the glucose metabolic rate of 824(thiCGE) were higher than those of the wild type strain (Fig. 5a). This may be attributed to the improved enzymes activities of glucose metabolism by reinforcing the VB1 biosynthesis, which was consistent with the previous research that VB1 supplement enhanced the metabolic flux and enzyme activity (phosphofructokinase) of glycolysis pathway (Xu et al. 2008). In addition, since 824(thiCGE) can produce sufficient energy from glucose metabolism due to the enhanced VB1 biosynthesis (Fig. 4b), the acids concentrations were decreased significantly (Fig. 5c). With the improved efficiency of glucose metabolism and ATP production, the butanol production of 824(thiCGE) was also enhanced

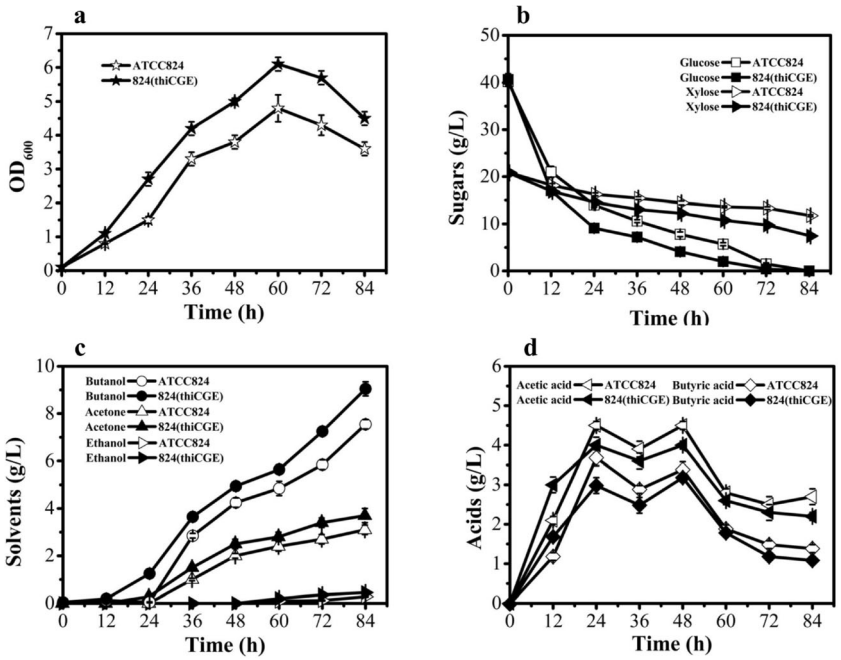

Fig. 7 Batch fermentation profiles of *C. acetobutylicum* ATCC 824 and 824(thiCGE) cultivated in VB1 free P2 medium with mixed sugars (40 g/L glucose and 20 g/L xylose). **a** The profiles of growth. **b** The profiles of sugars consumption. **c** The profiles of solvents production. **d** The profiles of organic acids production. The data are the means and standard deviations of three replicates

when compared with the wild-type strain (Fig. 5b). For example, the butanol titer in the VB1 free and normal P2 medium were 10.94 ± 0.08 g/L and 13.18 ± 0.23 g/L for 824(thiCGE), respectively, 13.0 ± 0.1% and 19.8 ± 0.6% higher than that of the wild-type strain (Table 2). It should be noted that the fermentation performances of 824(thiCGE) in VB1 free P2 medium were comparable to the wild-type strain in normal P2 medium, which means that the engineered strain 824(thiCGE) could produce adequate amounts of VB1 to replace exogenous VB1 addition in normal P2 medium (Table 2 and Table 3). Therefore, this strategy could reduce the investment of ABE fermentation and improve the economic benefit.

Pentose phosphate pathway was essential for xylose metabolism, and xylose utilization could be improved by overexpressing the genes involved in pentose phosphate pathway in *C. acetobutylicum* (Gu et al. 2009; Jin et al. 2014). As a coenzyme, VB1 also participates in the pentose phosphate pathway (Bazurto et al. 2015; Manzetti et al. 2014; Tittmann 2009). Therefore, it is expected that VB1 will have a positive effect on xylose metabolism. When fermentation was carried out in normal P2 medium using xylose as the substrate, the butanol productivity of *C. acetobutylicum* ATCC 824 reached 0.108 ± 0.004 g/L·h, exceeding that of in VB1 free P2 medium by more than 16.1 ± 3.7% (Table 3), demonstrating that VB1 also plays an important role in xylose metabolism. Furthermore, 824(thiCGE) produced 12.7 ± 1.2% and 14.9 ± 0.8% more butanol than the wide-type in VB1 free and normal P2 medium (Table 3), respectively. It is worth noting that when mixed sugars (glucose:xylose = 2:1) was used as the substrate in VB1 free P2 medium, the engineered strain 824(thiCGE) also showed better fermentation performance than the wild-type strain (Fig. 7). Both glucose and xylose utilization were improved by reinforcing the intracellular VB1 biosynthesis, although xylose consumption was still lagged behind glucose. As a result, the xylose metabolic rate and butanol titer of 824(thiCGE) were increased by 45.8 ± 1.9% and 20.4 ± 0.3% compared with the wild-type strain (Table 4). Similarly, the comparative transcriptomes analysis among the mutant strains with improved xylose utilization and the native strain of *Saccharomyces cerevisiae* showed that the most prominently changed (upregulated) genes were involved in VB1 biosynthesis (Zeng et al. 2017). Improving xylose utilization of *C. acetobutylicum* is of great significance to ABE fermentation with lignocellulosic biomass. Therefore, this metabolic engineering strategy may provide a new way for enhanced use of lignocellulosic biomass.

In this study, we have successfully constructed a robust engineering bacterium by reinforcing the VB1 biosynthetic pathway in *C. acetobutylicum* for the first time. The engineered strain 824(thiCGE) showed greatly improved performance grown on glucose in both VB1 free and normal P2 medium. What is more, improved xylose utilization was also observed when xylose or mixed sugars were used as the substrate. All these results demonstrated that this strategy will have some potential application value in ABE fermentation using simple medium and/or lignocellulosic biomass. On the other hand, the VB1 biosynthesis in 824(thiCGE) is still insufficient to reach the optimal condition (3 mg/L). Therefore, further improve the VB1 biosynthesis in *C. acetobutylicum* ATCC 824 is necessary in future research. To achieve this objective, the methods of

Table 4 Batch fermentation of *C. acetobutylicum* ATCC 824 and 824(thiCGE) using VB1 free P2 medium with mixed sugars in serum bottles

	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Butanol yield (g/g)	Butanol productivity (g/L/h)	Total solvent (g/L)	Max OD_{600}	Specific growth rate (h^{-1})	Total sugar consumption (g/L)	
											Glucose	Xylose
ATCC 824	3.13 ± 0.18	7.50 ± 0.19	0.28 ± 0.02	2.70 ± 0.20	1.41 ± 0.07	0.152 ± 0.002	0.090 ± 0.004	10.96 ± 0.40	4.8 ± 0.4	0.059 ± 0.001	40.23 ± 1.03	9.13 ± 0.21
824(thiCGE)	3.68 ± 0.30	9.03 ± 0.26	0.45 ± 0.04	2.22 ± 0.07	1.07 ± 0.12	0.167 ± 0.002	0.108 ± 0.005	13.15 ± 0.31	6.1 ± 0.2	0.061 ± 0.001	40.70 ± 1.16	13.31 ± 0.13

"±" average of three replicate

modifying promoter and regulatory parts or overexpressing the genes in chromosome instead of in plasmid can be used, which have been proved to be effective in improving gene expression (Shi et al. 2013; Yang et al. 2016).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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