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ORIGINAL ARTICLE

Gene modification of *Escherichia coli* and incorporation of process control to decrease acetate accumulation and increase L-tryptophan production

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Abstract Acetate is a primary inhibitory metabolite in cultures of *Escherichia coli*, and the production of both biomass and desired products are increased by reducing the accumulation of acetate. In this study, the accumulation of acetate during -tryptophan production was decreased by genetic modification of -tryptophan-producing strain (BCTRP) and optimization of the fermentation process. The mutant (BCTRPG), which has a deletion of the integral membrane permease IICB^{Glc} (*ptsG*), produces a higher concentration of -tryptophan than mutants with deletions of either phosphate acetyltransferase (*pta*) or *pta–ptsG*, due to the low accumulation of acetate and other byproducts, as well as high biomass production. The appropriate dissolved oxygen (DO) level, glucose feeding mode, and pH control strategy were applied to -tryptophan production using the BCTRPG mutant. The BCTRPG strain with optimized conditions resulted in a reduction in acetate accumulation (71.08% reduction to 0.72 g/L) and an increase in -tryptophan production (35.81% increase to 17.14 g/L) compared with the BCTRP strain in the original culture condition. Meanwhile, an analysis of the metabolic flux distribution indicated that the acetate synthesis flux decreased from 19.2% (original conditions) to 8.4% (optimized conditions), and the flux of tryptophan formation with the optimized conditions was 18.5%, which was 1.89 times higher than under the original conditions. This study provided the theoretical foundation and technical support for high-level industrialization production of -tryptophan.

Keywords *Escherichia coli* · L-Tryptophan · Acetate · Gene modification · Process control · Metabolic flux

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Introduction

L-Tryptophan is an important amino acid that is widely used in medicine and as a feed additive (Schmid et al. 2004). The most commonly used method for L-Tryptophan production is the direct fermentation of cheap renewable carbohydrates, such as sucrose or glucose (Liu et al. 2012). The majority of tryptophan is produced by microbial fermentation using *Corynebacterium glutamicum* or *Escherichia coli* (Ikeda 2006). L-Tryptophan production by *E. coli* has been extensively investigated, including the overexpression of key tryptophan biosynthesis genes, the disruption of genes important for repressing tryptophan formation, the modification of the tryptophan transport system, the reduction of acetate accumulation, and the optimization of feeding and cultivation conditions (Bongaerts et al. 2001; Shen et al. 2012; Cheng et al. 2012).

Acetate metabolism in *E. coli* plays an important role in the control of the central metabolism and bioprocess performance (Castaño-Cerezo et al. 2009), and the simplified model for the acetate biosynthesis pathway in *E. coli* is presented in Fig. 1

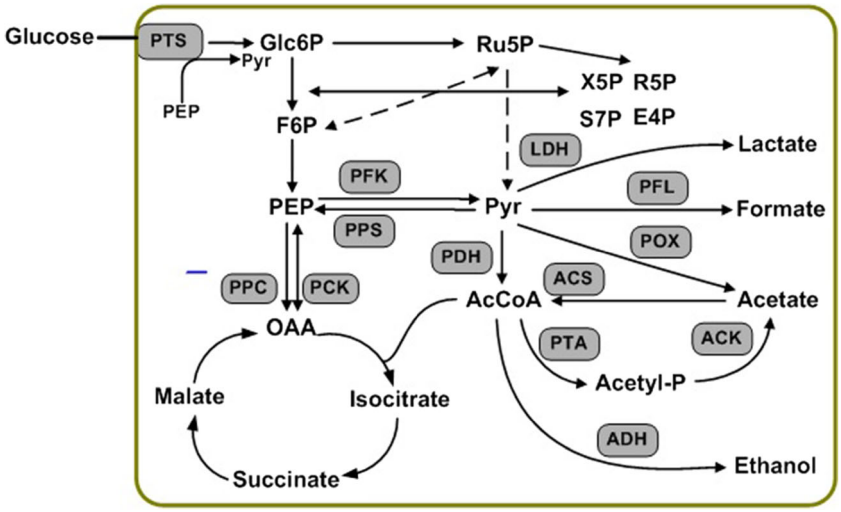

Fig. 1 Simplified model for the acetate biosynthesis pathway in *Escherichia coli*. The key enzymes for acetate synthesis involved are shown in the figure: PPS (phosphoenolpyruvate synthase), PFK (phosphofructokinase), PCK (phosphoenolpyruvate carboxykinase), PDH (pyruvate dehydrogenase), LDH (lactate dehydrogenase), PFL (pyruvate formate-lyase), POX (pyruvate oxidase), ACS (acetyl-CoA synthetase), PTA (phosphotransacetylase), ACK (acetate kinase)

(Gosset 2005; Castaño-Cerezo et al. 2009). The accumulation of acetate inhibits growth even at concentrations as low as 0.5 g/L (Nakano et al. 1997); this is one of the obstacles to increasing productivity and attaining high product yields (Åkesson et al. 2001). Acetate accumulation has been reduced by manipulating strains and cultivation conditions (Eiteman and Altman 2006). In *E. coli*, acetate is mainly synthesized by the phospho-transcetylase (Pta)–acetate kinase (AckA) pathway, which uses acetyl coenzyme A as a substrate (De Anda et al. 2006). The deletion of *pta* decreases acetate accumulation and increases L-Tryptophan production (Wang et al. 2013). The acetate accumulation in a culture of the Δ*pta* mutant was 14-fold lower than in a culture of wild-type *E. coli* (Castaño-Cerezo et al. 2009). *Escherichia coli* excretes acetate in response to an oversupply of pyruvate from the main glucose transport system, i.e., the carbohydrate phosphotransferase system (PTS); this suggests that acetate excretion is due to an imbalance in metabolite flux (Chang et al. 1999). When glucose is in excess, the tricarboxylic acid cycle (TCA) cycle is limited, acetyl-CoA accumulates, and 15–30% is excreted as acetate (Castaño-Cerezo et al. 2009). PTS mutants produce substantially lower levels of acetate compared with wild-type strains (Báez-Viveros et al. 2007). The glucose-specific complex of PTS (II^{Glc}) is composed of a soluble enzyme (IIA^{Glc}) and an integral membrane permease ($IICB^{Glc}$), which are encoded by *crr* and *ptsG*, respectively (Gosset 2005). The *ptsG* mutant is cultured in a complex medium containing glucose, and there is a reduction in acetate secretion and an increase (>50%) in the levels of a recombinant protein (Gosset 2005; Sigala et al. 2009). The inactivation of PTS increases the supply of phosphoenolpyruvate (PEP), which, in turn, increases the metabolic flux of aromatic compounds (Lu et al. 2012).

Acetate accumulation under aerobic conditions depends on the bacterial strain and typically occurs during high growth rates and/or low oxygen conditions (Noronha et al. 2000). Increased acetate accumulation at lower dissolved oxygen (DO) levels is a result of diminished TCA activity, altered transcription levels of genes associated with glucose and acetate metabolism (Phue and Shiloach 2005), and low concentrations of acetate that accumulate at high DO levels. In fed-batch cultures, which are often preferred, the feed rate of the carbon source (usually glucose) can be manipulated to restrict acetate formation (Eiteman and Altman 2006). It is possible to avoid acetate accumulation by keeping the feed rate sufficiently low. However, choosing a feed rate that is unnecessarily low will result in a low growth rate, a long cultivation time, and low productivity levels (Cheng et al. 2012). Utilizing a probing feeding technique using a standard DO probe for the cultivation of recombinant strains gives the highest feed rate possible while avoiding limitations from overflow metabolism and oxygen transfer. Thus, this maximizes bioreactor productivity by maintaining low concentrations of acetate and glucose (Åkesson et al. 2001). pH is an important parameter in *E. coli* cultivation. Several enzymes related to the production of L-Tryptophan and acetate are induced at the pH extremes, including the proteins encoded by *aroK*, *tnaA*, *tnaB*, *pta*, *sucB*, and *sucC* (Stancik et al. 2002; Ikeda 2006). Maintaining an optimal pH level during L-Tryptophan fermentation can decrease acetate accumulation and increase L-Tryptophan production (Cheng et al. 2013).

In this study, we decreased acetate accumulation during L-Tryptophan production by genetically modifying a L-Tryptophan-producing strain and optimizing the cultivation conditions. Three recombinant strains, BCTRPP (BCTRP Δ*pta*), BCTRPG (BCTRP Δ*ptsG*), and BCTRPPG (BCTRP Δ*pta* Δ*ptsG*), were generated from BCTRP. We used these mutants to investigate the effect of deletions of *pta* and/or *ptsG* on acetate accumulation and L-Tryptophan production, and selected a recombinant strain that had low acetate and high L-Tryptophan levels for further study. We employed different DO concentrations, feedback controls on glucose feeding, and sequential pH adjustments to reduce acetate accumulation and improve L-Tryptophan production. In addition, we analyzed the metabolic flux distribution under different

culture conditions to explain the mechanisms driving low acetate accumulation and high production of L-Tryptophan.

Materials and methods

Bacterial strains, plasmids, and primers

All bacterial strains, plasmids, and primers used in this study are listed in Table 1. The strain BCTRP as the parent strain was used to produce L-Tryptophan in this study, which was obtained from a previous work in our laboratory and stored at the Culture Collection of Shandong Binzhou Animal Science and Veterinary Medicine Academy. In the earlier study, the mutant producing high concentrations of L-Tryptophan was derived from *E. coli* W3110 by ultraviolet mutagenesis, and the sequence analysis of the mutant confirmed replacement of the residue Pro 150 with Leu in *aroG* and the residue Met 293 with Thr in *trpE*, which indicated that the genes $aroG^{fbr}$ and $trpE^{fbr}$ existed in the mutant (Jiang et al. 2000; Cheng et al. 2012; Gu et al. 2012); the plasmid pSTV-032 was obtained by inserting the genes $aroG^{fbr}$ and $trpE^{fbr}DCBA$ into the plasmid pET-32a, and the strain BCTRP was constructed by transforming the plasmid pSTV-032 into the *E. coli* W3110 with deletion of the *trpR–tnaA* genes.

Media

The media used for generating and propagating recombinant strains were prepared according to published procedures (Liu et al. 2012). The seed medium consisted of: 20 g/L glucose, 15 g/L yeast extract, 10 g/L $(NH_4)_2SO_4$, 0.5 g/L sodium citrate, 5 g/L $MgSO_4{\cdot}7H_2O$, 1.5 g/L KH_2PO_4, 0.015 g/L $FeSO_4{\cdot}7H_2O$, and 0.1 g/L vitamin B_1. The fermentation medium for producing L-Tryptophan consisted of: 20 g/L glucose, 1 g/L yeast extract, 4 g/L $(NH_4)_2SO_4$, 2 g/L sodium citrate, 5 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L KH_2PO_4, and 0.1 g/L $FeSO_4{\cdot}7H_2O$. The pHs of all media were adjusted to 7.0 with 4 M NaOH.

Culture conditions

The culture conditions used for generating and propagating recombinant strains were controlled according to published procedures (Liu et al. 2012). Fermentations were carried out in a 30-L fermenter. A 500-mL baffled flask containing 30 mL of seed medium was inoculated with a single colony of each

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

Name	Characteristics	Source
Strains		
BCTRP	*E. coli* Δ*trpR* Δ*tna*, $aroG^{fbr}trpE^{fbr}DCBA$ Am^R	Lab collection
BCTRPP	Derived from BCTRP, but Δ*pta*	This study
BCTRPG	Derived from BCTRP, but Δ*ptsG*	This study
BCTRPPG	Derived from BCTRP, but Δ*pta* and Δ*ptsG*	This study
Plasmids		
pKD46	Am^R, λ Red-expressing vector	Datsenko and Wanner (2000)
pKD3	Cm^R, template vector	Datsenko and Wanner (2000)
pCP20	Am^R, Cm^R, FLP-expressing vector	Datsenko and Wanner (2000)
Primers		
pta-P1[a]	5′-GCTGGCGGTGCTGTTTTGTAACCCGCCAAATCGGCGGTAACGAAAGAGGATAAACCTTGA GCGATTGTGTAGGCTGGAG-3′[b]	This study
pta-P2[a]	5′-TAGTGATTATTTCCGGTTCAGATATCCGCAGCGCAAAGCTGCGGATGATGACGAGATAAC GGCTGACATGGGAATTAGC-3′[b]	This study
pta-P3	5′-GTTTCGGCAAATCTGGTTTCATC-3′	This study
pta-P4	5′-TGGTAAGTATGCAAAGTGGGATGG-3′	This study
ptsG-P1[a]	5′-ATGTTTAAGAATGCATTTGCTAACCTGCAAAAGGTCGGTAAATCGCTGATGCTGCCTTGA GCGATTGTGTAGGCTGGAG-3′[b]	This study
ptsG-P2[a]	5′-TTAGTGGTTACGGATGTACTCATCCATCTCGGTTTTCAGGTTATCGGATTTAGTACTAAC GGCTGACATGGGAATTAGC-3′[b]	This study
ptsG-P3	5′-AGTTGAAACGTGATAGCCGTC-3′	This study
ptsG-P4	5′-AACGTGGAAGGTTCTATCGTCTAC-3′	This study

[a] The underlined sequences indicate 56-nt homology extensions of a target knockout gene

test strain and cultivated at 36 °C with shaking (200 rpm) for 12 h. Thirty milliliters of this culture was added aseptically to a 5-L seed fermenter (Biotech-2002 Bioprocess controller, Baoxing, Shanghai, China) containing 3 L of seed medium and cultivated at 36 °C for 16 h. The culture grown in the seed fermenter was used to aseptically inoculate (10% v/v) 18 L of production medium in a 30-L fermenter. The temperature and DO levels were maintained at 36 °C and 20%, respectively. 25% ammonium hydroxide (w/w) was used to maintain the pH at 7.0. When the initial glucose was depleted, a glucose solution (80% w/v) was delivered to the fermenter to maintain the specified experimental requirements.

Construction of the strains

Single- and multi-gene deletion mutants were constructed as previously described (Datsenko and Wanner 2000; Liu et al. 2012). The deletion of *pta* in BCTRP was performed using the Red helper plasmid, pKD46. The appropriate DNA fragment was obtained by polymerase chain reaction (PCR) using the *pta*-P1 and *pta*-P2 primers with the helper plasmid pKD3. To eliminate the Cm^R gene from the integrated locus, the cells were transformed with the pCP20 plasmid carrying the FLP recombinase gene. All test PCRs were performed using the *pta*-P3 and *pta*-P4 primers. The disruption of *ptsG* in the BCTRP and/or BCTRPP mutants was performed using the same method used to disrupt *pta* using the *ptsG*-P1, *ptsG*-P2, *ptsG*-P3, and *ptsG*-P4 primers.

Analysis of fermentation products

The biomass (dry cell weight) level and L-Tryptophan concentration in the fermentation broth were determined as described previously (Liu et al. 2012). The concentrations of glucose and lactate were monitored using an SBA-40E biosensor analyzer (Biology Institute of Shandong Academy of Sciences, Jinan, China). Concentrations of acetate and pyruvate were measured using a BioProfile 300A biochemical analyzer (Nova Biomedical, Waltham, MA, USA).

Analysis of metabolic flux distribution

The metabolic flux distribution of tryptophan during the later fermentation period of L-Tryptophan production was calculated by MATLAB (MathWorks, Natick, MA, USA), based on an analysis of metabolic flux balance and stoichiometry (Báez-Viveros et al. 2007; Zhao et al. 2016).

Statistical analysis

All experiments were conducted in triplicate. The data were averaged and are presented as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test was used to determine significant differences. Statistical significance was defined as $P < 0.05$.

Results

Construction of the mutants

The knockouts of *pta* and *ptsG* in *E. coli* were confirmed by colony PCR using the *pta*-P3–*pta*-P4 and *ptsG*-P3–*ptsG*-P4 primers, respectively. The lengths of the fragments detected agreed with their theoretical lengths; thus, we confirmed that *pta* and/or *ptsG* deletion mutants were successfully constructed.

L-Tryptophan production with different strains

Accumulation of byproducts

The mutants had lower acetate accumulation than BCTRP (Fig. 2a). Acetate accumulation by BCTRPG (1.35 g/L) was lower than that in BCTRP (2.49 g/L) and BCTRPP (1.85 g/L) by 45.78% and 27.03%, respectively. The acetate accumulation of BCTRPPG (1.30 g/L) was the lowest. The acetate consumption rate of BCTRP was lower than that of BCTRPG, but higher than that of the *pta* mutant (Fig. 2b). The acetate consumption rate of BCTRPPG was lower than that of BCTRPG due to the knockout of *pta*. The BCTRPP strain accumulated the highest levels of pyruvate (2.01 g/L) and lactate (2.84 g/L) during the growth phase; these levels were 4.17 and 2.03 times higher than those of BCTRP, respectively (Fig. 2c, d). BCTRPG accumulated 0.28 g/L pyruvate and 0.71 g/L lactate, which were 41.72% and 49.31% lower than BCTRP, respectively. The pyruvate and lactate concentrations in the BCTRPPG culture were lower than those in the BCTRP and BCTRPP cultures, but higher than those in the BCTRPG culture during the early fermentation period.

Biomass and production of L-Tryptophan

Though the deletion of *pta* and/or *ptsG* decreased the growth rate of the strains, the biomass levels of BCTRPP and BCTRPG were higher than that of BCTRP (Fig. 3). The biomass obtained in the BCTRPG culture was the highest of all strains (24.22 g/L); this is 14.08% higher than the BCTRP strain (21.23 g/L). The biomass of the BCTRPPG strain was the lowest (19.61 g/L); this was 7.63% lower than the BCTRP strain. The deletion of *pta* and/or *ptsG* increased the capacity for L-Tryptophan production (Fig. 3). The L-Tryptophan production of the BCTRPG strain was the highest of those tested in this study (14.53 g/L), and was 15.13% higher than that of

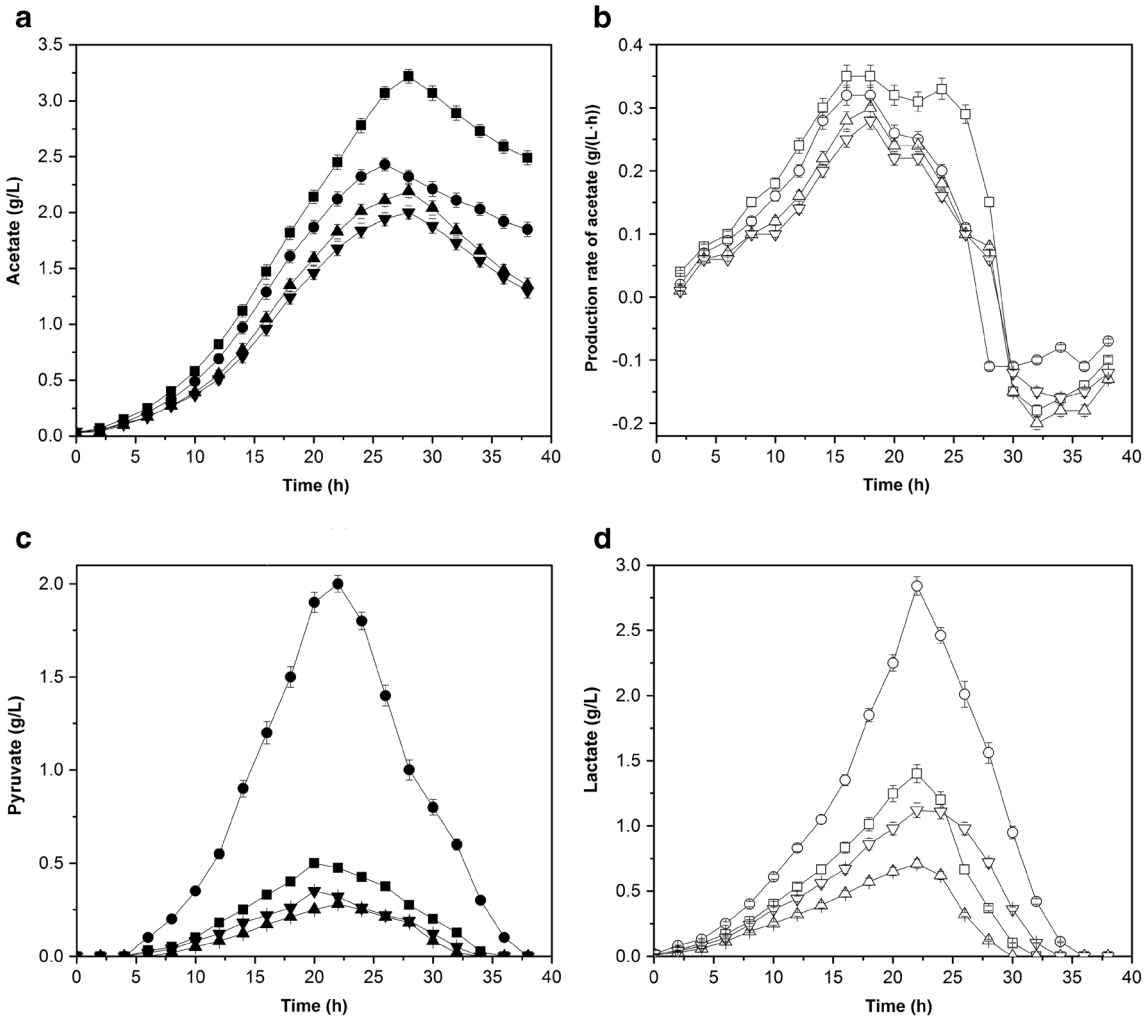

Fig. 2 Effect of genetic modification on the accumulation of byproducts during -tryptophan production. *Squares* BCTRP, *circles* BCTRPP, *upright triangles* BCTRPG, and *inverse triangles* BCTRPPG. The absolute value of the data in **b** less than zero indicates the consumption rate of acetate ($P < 0.05$)

the BCTRP strain (12.62 g/L). The BCTRPP and BCTRPPG mutants produced 13.72 g/L and 13.14 g/L of L-Tryptophan, respectively.

L-Tryptophan production with different DO levels

The DO in BCTRPG cultures was controlled at 10%, 20%, and 30% (Table 2). The consumption of acetate, biomass, and L-Tryptophan production increased with higher DO concentration; meanwhile, the accumulation of acetate decreased as DO concentrations increased. The highest biomass (25.22 g/L) and L-Tryptophan (15.33 g/L) levels were obtained when DO was maintained at 30%; these levels are 8.17% and 15.11% above those obtained at a DO level of 10%, respectively. Acetate accumulations were 1.84 g/L, 1.58 g/L, and 1.12 g/L for DO concentrations of 10%, 20%, and 30%, respectively. At 30% DO, 0.92 g/L acetate was reused for L-

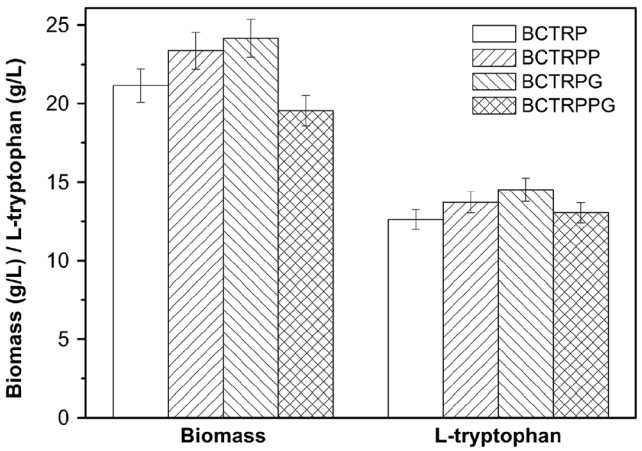

Fig. 3 Effect of genetic modification on biomass levels and the production of -tryptophan ($P < 0.05$)

Table 2 -Tryptophan production using BCTRPG under different dissolved oxygen (DO) levels

Kinetic parameters	DO levels		
	10%	20%	30%
Biomass (g/L)	23.31 ± 0.58	24.23 ± 0.52	25.22 ± 0.53
-Tryptophan (g/L)	13.31 ± 0.28	14.51 ± 0.31	15.33 ± 0.32
Maximum concentration of acetate (g/L)	2.54 ± 0.06	2.33 ± 0.05	2.04 ± 0.06
Consumption concentration of acetate (g/L)	0.70 ± 0.02	0.75 ± 0.03	0.92 ± 0.03
Accumulation of acetate (g/L)	1.84 ± 0.05	1.58 ± 0.04	1.12 ± 0.04

Tryptophan production, at 20% 0.75 g/L was reused, and at 10% 0.70 g/L was reused.

L-Tryptophan production with glucose feedback feeding

In the early period of L-Tryptophan production using the BCTRPG strain, the DO level was maintained at 30% by adjusting the agitation and aeration rates, and the oxygen response to feed pulses was achieved. In the later part of the cultivation, the maximum rates of agitation and aeration were unable to satisfy the oxygen requirement; thus, the DO level was maintained at 30% by adjusting the feed rate of glucose (Fig. 4). The glucose concentration was maintained at a low level (0.20 g/L) and the acetate accumulation (0.98 g/L) was 12.50% lower than when glucose feedback feeding was not applied (Fig. 4a). The biomass and L-Tryptophan production levels were 1.02 and 1.07 times higher (25.73 g/L and 16.34 g/L), respectively, than when glucose feedback feeding was not used (Fig. 4b).

L-Tryptophan production with sequential pH adjustment

We also examined the effects of pH control (6.5 from 0 to 12 h, 6.8 from 12 to 24 h, and 7.2 from 24 to 38 h) on L-Tryptophan production by the mutant BCTRPG at a DO level of 30% using glucose feedback feeding (Fig. 5). The acetate accumulation was 0.72 g/L, which was 26.53% lower than when sequential pH adjustment was not employed (Fig. 5a). The maximum biomass and L-Tryptophan levels increased by 1.98% and 5.12% to 26.24 g/L and 17.14 g/L, respectively, compared with the levels when the pH was maintained at 7.0 (Figs. 5b and 4b).

Metabolic flux distribution under different conditions

The flux of carbon was redistributed by the deletion of *ptsG* and optimization of culture conditions (Fig. 6). With the deletion of *ptsG* and the optimization of culture conditions, more carbon flux entered the pentose phosphate (PP) pathway, the flux of byproducts decreased, and tryptophan biosynthesis flux increased. The flux of acetate with the optimized conditions (i.e., the mutant BCTRPG and optimized culture

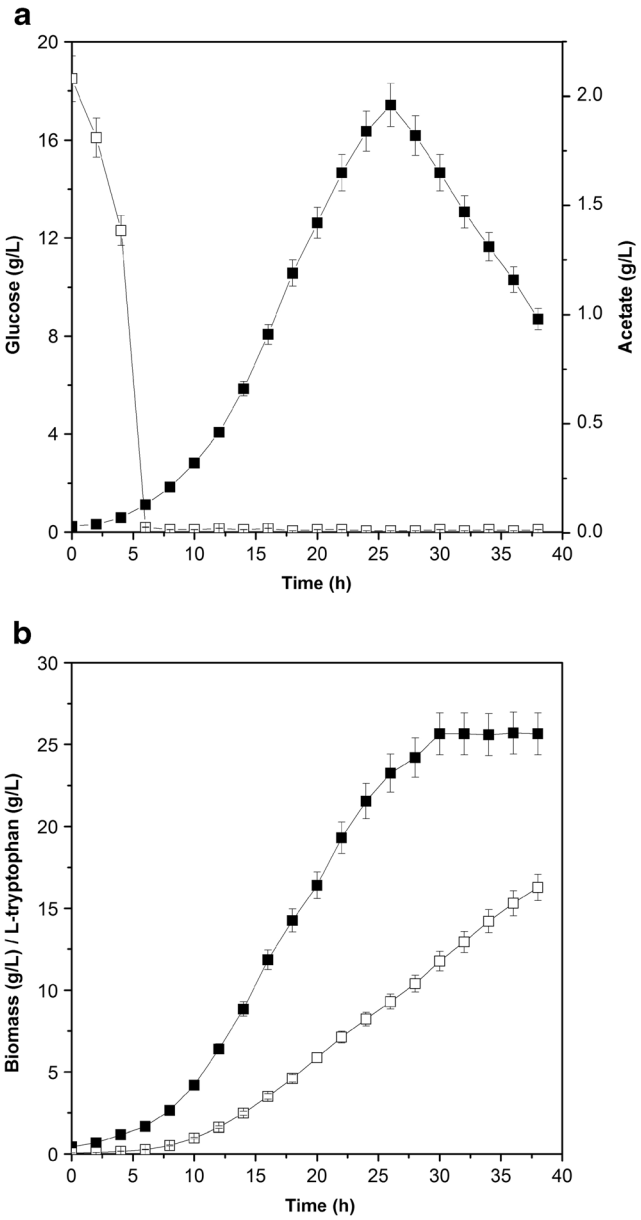

Fig. 4 -Tryptophan production in a fermentation with a dissolved oxygen (DO) level of 30% and the application of glucose feedback feeding. **a** *Open squares* concentration of glucose and *filled squares* acetate levels. **b** *Filled squares* biomass, and *open squares* -tryptophan levels ($P < 0.05$)

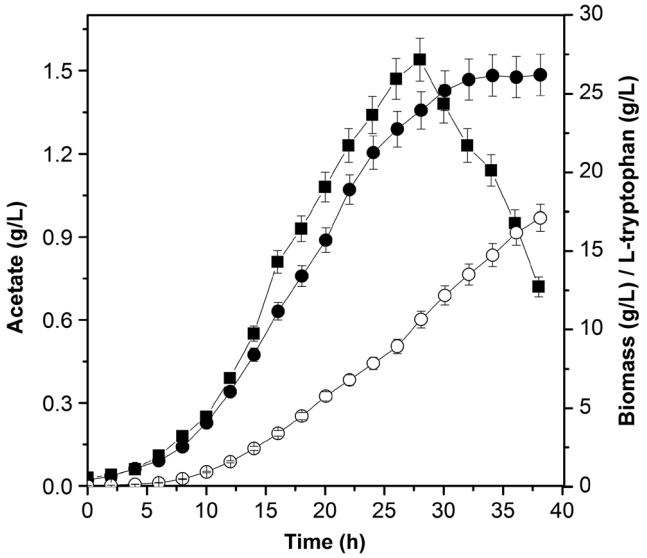

Fig. 5 -Tryptophan production in a fermentation with a DO level of 30%, glucose feedback feeding, and sequential pH adjustments. *Filled squares* accumulation of acetate, *filled circles* biomass, and *open circles* -tryptophan levels ($P < 0.05$)

conditions) was 8.4%, which was 56.25% lower than under the original conditions (i.e., the strain BCTRP and original culture conditions). The carbon flux of tryptophan biosynthesis with the optimized conditions was 18.5%, which was 1.89 times higher than under the original conditions.

Discussion

Gene modification of *E. coli*

The deletions of *pta* and/or *ptsG* effectively decreased the excretion of acetate, and the lowest levels of acetate accumulation were observed in the BCTRPPG mutant because of the deletion of *pta–ptsG* (Fig. 2a) (Castaño-Cerezo et al. 2009; Sigala et al. 2009). Acetate consumption is achieved via its conversion back to acetyl-CoA by acetyl-CoA synthetase (Acs) and the reverse action of Pta–AckA (Phue and Shiloach 2004). Acs permits *E. coli* to utilize acetate as a

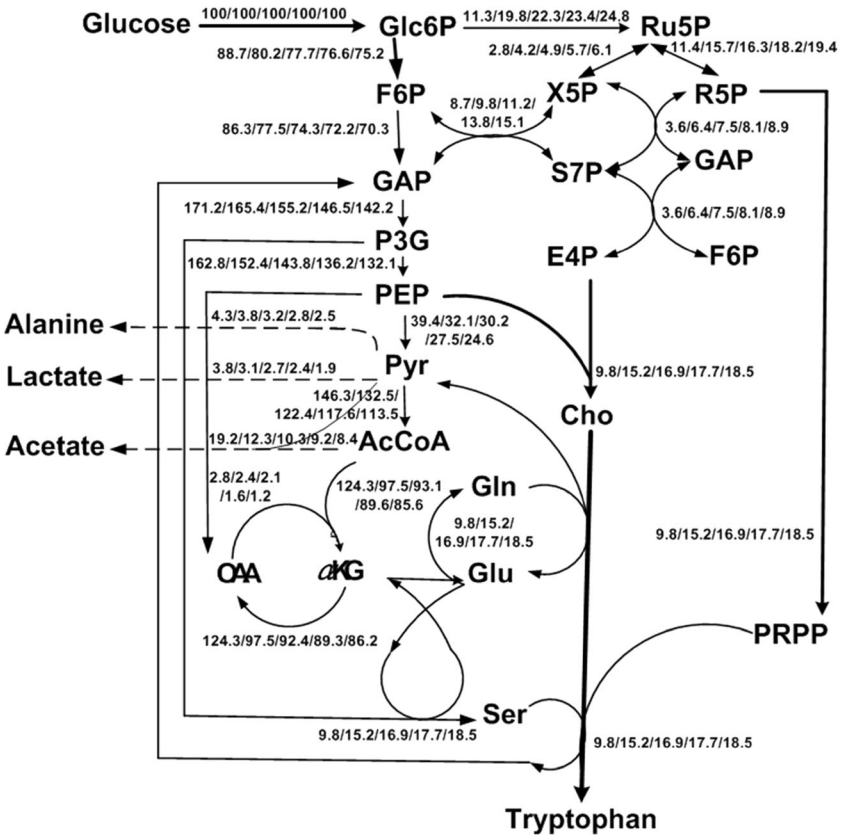

Fig. 6 The metabolic flux distribution of -tryptophan biosynthesis under different culture conditions during the later fermentation periods (30–38 h). -Tryptophan production with different culture conditions are shown as: BCTRP with original culture conditions (first value); BCTRPG with original culture conditions (second value); BCTRPG with 30% DO (third value); BCTRPG with 30% DO and glucose feedback feeding (fourth value); and BCTRPG with 30% DO, glucose feedback feeding, and sequential pH adjustment (fifth value). Metabolite abbreviations: Glc6P (glucose 6-phosphate), F6P (fructose 6-phosphate), GAP (glyceraldehyde 3-phosphate), P3G (3-phosphoglycerate), PEP (phosphoenolpyruvate), Pyr (pyruvate), AcCoA (acetyl coenzyme A), Ru5P (ribulose 5-phosphate), X5P (xylulose 5-phosphate), S7P (sedoheptulose 7-phosphate), E4P (erythrose 4-phosphate), Cho (chorismate), PRPP (5-phosphoribosyl pyrophosphate), OAA (oxaloacetate), αKG (α-ketoglutarate), Gln (glutamine), Glu (glutamate), and Ser (serine)

source of energy to create building blocks that enable survival during periods of starvation (Báez-Viveros et al. 2007). The overexpression of Acs in *E. coli* results in a significant decrease in acetate accumulation (Lin et al. 2006). The deletion of *pta* causes a decrease in Acs expression and activity, which partially explains the low acetate consumption rate (Castaño-Cerezo et al. 2009). The inactivation of *ptsG* is an effective strategy for reducing glucose uptake, which leads to a decrease in acetate excretion (Tang et al. 2013). The *ptsG* mutant exhibited lower acetate accumulation and a higher acetate consumption rate due to the up-regulation of genes related to acetate consumption and transport (i.e., the *acs–actP* operon) (Fig. 2b) (Báez-Viveros et al. 2007). The reduction in acetate flux in the *pta* mutant was redirected to the excretion of pyruvate, D-lactate, and -glutamate (Chang et al. 1999). Pyruvate accumulates in *E. coli* cells when *pta* is deleted because the fermentation profile is completely altered, and this leads to an increase in lactate concentration (Chang et al. 1999; Zhu and Shimizu 2005). In the mutant with a lesion in *ptsG*, lower concentrations of byproducts accumulated because of the reduced glucose uptake rate (Fig. 2c, d) (Postma et al. 1993; Picon et al. 2005).

The genetic modification of strains impact on the cell growth rate due to alterations to the normal cell metabolism (Huang et al. 2011). The mutants with *pta* and/or *ptsG* deletions showed lower growth rates than BCTRP. The deletion of *pta–ptsG* was detrimental to the growth of the strain, and resulted in the lowest biomass. BCTRPG had the highest growth rate because glucose is transported by other PTS complexes, such as EII^{Man} and EII^{Bgl} (Phue and Shiloach 2004). Higher biomass and L-Tryptophan levels were obtained in BCTRPP and BCTRPG cultures due to the decreased acetate levels (Fig. 3) (Dodge and Gerstner 2002). The deletion of *ptsG* decreased acetate flux from 19.2% to 12.3% (Fig. 6) (Contiero et al. 2000; Dittrich et al. 2005). Inactivation of PTS is a common strategy for increasing the PEP supply to improve metabolic flux toward desired products (Lu et al. 2012). The tryptophan flux in the *ptsG* deletion mutant was 15.2%, which was 55.10% higher than that of the BCTRP strain (Fig. 6). The highest L-Tryptophan levels were obtained in the BCTRPG strain; this indicates that the *ptsG* deletion mutant is the better strain for L-Tryptophan production.

Different DO levels

Both the TCA cycle and acetate excretion were affected by the transition from unlimited to limited supply of oxygen. This alters cellular metabolism and protein production capability (Phue and Shiloach 2005). Acetate accumulation under aerobic conditions typically occurs with high growth rates and/or low oxygen concentrations (Noronha et al. 2000; Cheng et al. 2012). The transcription levels of key genes for acetate formation and assimilation are affected by DO conditions (Phue and Shiloach 2005). The transcription of the gluconeogenesis (*pckA*, *ppsA*) and anaplerotic pathway (*ppc*, *sfcA*) genes was lower at low DO concentrations than that at high DO concentrations; this contributed to the accumulation of pyruvate and acetyl-CoA, and caused acetate accumulation through the Pta–AckA and PoxB pathways (Fig. 1) (Phue and Shiloach 2005). Thus, less acetate was accumulated at higher DO levels than at lower DO levels (Table 2). The acetate synthesis flux at 30% DO was 10.3%, which was 16.26% lower than at a DO of 20% (Fig. 6). Lower transcription levels of Acs are an indication of lower acetate uptake, and more acetate was consumed at higher DO levels than at lower DO levels. Higher expression levels of phosphoenolpyruvate synthase (Pps) at higher DO levels increased the availability of PEP, a major precursor for aromatic amino acid production (Shen et al. 2012). The flux of tryptophan biosynthesis at 30% DO (16.9%) was 11.18% higher than that at 20% DO; this resulted in increased L-Tryptophan production (Table 2 and Fig. 6).

Glucose feedback feeding

A number of feeding strategies were designed to reduce or avoid acetate formation and avoid either overflow metabolism or anaerobic conditions (Åkesson et al. 2001; Eiteman and Altman 2006). The online detection of overflow metabolism was enacted using a standard DO probe, and a simple feedback algorithm was used to adjust the glucose feed rate to avoid overflow metabolism while maintaining a high glucose uptake rate (Åkesson et al. 2001). A balance between oxygen transfer and oxygen uptake can be achieved by manipulating the feed rate to maintain a constant DO concentration and preclude anaerobic conditions (Riesenberg et al. 1990). Acetate accumulation decreased when glucose feedback feeding was employed; this was expected based on the low rate of acetate excretion at low glucose concentrations and aerobic conditions (Phue and Shiloach 2005; Cheng et al. 2012). The flux of acetate synthesis with glucose feedback feeding was 9.2%, which was 10.68% lower than when glucose feedback feeding was not employed (Fig. 6). There were no indications of overflow metabolism during the entire fermentation process (Åkesson et al. 2001). With glucose feedback feeding, the carbon flux for tryptophan biosynthesis increased to 17.7% and high levels of L-Tryptophan were produced (Figs. 4 and 6). High biomass and L-Tryptophan levels were obtained with application of this feeding strategy due to the low acetate accumulation and high DO level (Fig. 4) (Phue and Shiloach 2005; Cheng et al. 2012).

pH control strategy

The effects of pH are complex because pH influences other culture conditions, such as oxygenation, growth phase, and

metabolite composition (Stancik et al. 2002). Higher biomass and L-Tryptophan production are obtained with a sequential pH adjustment (Cheng et al. 2013). The *sucB* and *sucC* genes are induced in low pH cultures, which should result in the utilization of the high proton potential and increase the capacity of the TCA cycle (Stancik et al. 2002). This should lead to a decrease in pyruvate and acetate concentrations (Chang et al. 1999). Acetate flux decreased to 8.4% when sequential pH adjustment was employed (Fig. 6). The key gene for acetate synthesis, *pta*, is induced at high pH; however, there is low acetate accumulation because of the low transcriptional levels when DO levels are high and metabolic flux through glycolysis due to the deletion of *ptsG* is low (Fig. 1) (Phue and Shiloach 2005; De Anda et al. 2006). The activity of shikimate kinase I (AroK), which is involved in the synthesis of aromatic amino acids, is increased at high pH and is beneficial to L-Tryptophan synthesis because it can increase the formation of key precursors for L-Tryptophan biosynthesis (Fig. 5) (Stancik et al. 2002; Shen et al. 2012). Thus, more PEP and erythrose 4-phosphate (E4P) are supplied for tryptophan formation; this is evident by the increase in tryptophan biosynthesis flux to 18.5% (Fig. 6). Acetate excretion was lower and biomass and L-Tryptophan production were higher during L-Tryptophan production using the sequential pH adjustment.

We show that a *ptsG* deletion mutant produces higher levels of L-Tryptophan than *pta* or *pta–ptsG* deletion mutants. A DO of 30% and sequential pH adjustment with the BCTRPG strain using glucose feedback feeding resulted in an acetate accumulation of 0.72 g/L, 26.24 g/L of biomass, and 17.14 g/L of L-Tryptophan. These levels were 71.08% lower, 23.60% higher, and 35.81% higher, respectively, than the BCTRP strain under the original fermentation conditions. In addition, an analysis of metabolic flux distribution indicated that acetate flux with the optimized conditions was 8.4%, which is 56.25% lower than under the original conditions; meanwhile, the flux of tryptophan production increased to 18.5%, which was 1.89 times higher than under the original conditions.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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

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