Calcium Carbonate Addition Improves L-Methionine Biosynthesis by Metabolically Engineered Escherichia coli W3110-BL

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L-Methionine (L-Met) is a sulfur-containing amino acid, which is one of the eight essential amino acids to human body. In this work, the fermentative production of L-Met with genetically engineered Escherichia coli W3110-BL in a 5-L fermentor was enhanced through supplement of Ca2+ into the fermentation medium. With the addition of 30 g/L calcium carbonate (CaCO3), the titer of L-Met and yield against glucose reached 1.48 g/L and 0.09 mol/mol glucose, 57.45% higher than those of the control, respectively. The flux balance analysis (FBA) revealed that addition of CaCO3 strengthened the tricarboxylic acid cycle and increased the intracellular ATP concentration by 39.28%. The re-distribution of carbon, ATP, and cofactors flux may collaborate to improve L-Met biosynthesis with E. coli W3110-BL. The regulation of citrate synthase and oxidative phosphorylation pathway was proposed to be important for overproduction of L-Met. These foundations provide helpful reference in the following metabolic modification or fermentation control for further improvement of L-Met biosynthesis.

Keywords: L-methionine, metabolic flux, Escherichia coli, calcium carbonate, flux balance analysis

INTRODUCTION

Methionine (Met) is one of the eight essential amino acids of humans. It is extensively applied in the fields of pharmaceutics, food, and feed. Met can be also used as an important participant in the synthesis of S-adenosyl methionine (SAM), a common methyl-group donor involved in the biosynthesis of nucleic acids, phospholipids, proteins, epinephrine, melatonin, creatine, and other molecules. Along with L-lysine, Met is a dominant amino acid used in animal feed (Noftsger et al., 2005). In recent years, the global Met market for animal feed has been substantially increased with the rapid growing consumption of meat and milk products.

Currently, microbial fermentation with auxotrophic mutants or genetically engineered strains for production of Met has attracted more attentions than chemical synthesis, which exhibits disadvantages of environmental unfriendliness, strict reaction conditions and impure product with racemic mixtures. The pathway for L-methionine (L-Met) synthesis is well studied in many
microorganisms (Krömer et al., 2006). It is a multibranched and multilevel regulated biosynthetic pathway (Huang et al., 2018). L-Met biosynthesis derived from glucose with the canonical metabolic route requires 18 mol ATP, and L-Met is thereby known as the most expensive amino acid in terms of consumed mol of ATP per molecule produced (Kalita et al., 2013). Large amount of strategies have been developed to improve L-Met fermentative production, as for instance, traditional breeding (Kase and Nakayama, 1975; Kumar et al., 2003), genetical modification (Usuda and Kurahashi, 2005; Park et al., 2007; Huang et al., 2017, 2018), and fermentation optimization (Anakwenze et al., 2014; Zhou et al., 2019). However, the industrial preparation of L-Met by fermentation approach is still restricted since the metabolic flux is dispersed and its regulation is complicated. It is therefore of particular importance to identify the potential factors affecting the L-Met over-production.

In our previous study, based on the comprehensive analysis of feedback inhibition, synthetic bottlenecks, and cell growth repression in L-Met biosynthesis, a recombinant L-Met-producing strain Escherichia coli IJAHFEBL/pA⁺H (shortly named as E. coli W3110-BL) was constructed via deletion of metJ for partial inactivation of the L-Met import system MetD, metJ for elimination of negative transcription regulation by L-Met, and lysA for blocking the biosynthesis of the by-product L-lysine (Huang et al., 2017); additionally, the expression of metH encoding B12-dependent homocysteinase-N5-methylethryhaflolate transmethylase (MetH), metF encoding 5,10-methylenetetrahydroflololate reductase (MetF), cysE encoding serine acetyltransferase (CysE), and metB encoding cystathionine gamma-synthase (MetB)/bifunctional aspartate kinase/homoserine dehydrogenase II (MetL) were enhanced by replacing their native promoters on the chromosome by a strong promoter trc, respectively (Huang et al., 2017, 2018). The homoserine O-succinyltransferase with reduced feedback sensitivity to SAM and L-Met, as well as the L-Met efflux transporter were overexpressed by constructing a recombinant expression plasmid pA⁺H, of which the genes metA⁺br and yjeH were inserted into the plasmid pTrc99A, respectively (Huang et al., 2018). As a result, the L-Met production was significantly enhanced compared with the original strain E. coli W3110.

In order to further enhance the L-Met fermentation titer of this recombinant strain, the fermentation conditions need to be additionally optimized by process engineering. As is well-known, inorganic salts (minerals) are essential elements in the nutrition and are required for almost all living things. Inorganic salts starvation or limitation in microbial cells will lead to critical stress response (Wiesenberger et al., 2007). It has been demonstrated that supplement of optimal mineral salts (for example K₂HPO₄, MgSO₄·7H₂O, NaCl, CaCO₃, FeSO₄·7H₂O, MnCl₂·7H₂O, and ZnSO₄) in the fermentation medium exhibited positive effects on various fermentation product, such as vitamins (Oraei et al., 2018), amino acids (Zhou et al., 2019), antibacterial metabolite (Jacob et al., 2017), and polypeptide or enzyme (Zhou et al., 2014; Cheng et al., 2019). The bivalent metals including Zn²⁺, Mg²⁺, and Ba²⁺ at trace concentration (mg/L) were reported to play an important role to stimulate L-Met production with Bacillus thuringiensis EC1. After optimization of their concentrations, an improved L-Met yield of 3.18 g/L was achieved (Anakwenze et al., 2014). The effect of another bivalent metal calcium ion (Ca²⁺) on cell growth, morphology, and accumulation of target metabolites has also been reported by numerous researchers (Robson et al., 1991; Pera and Callieri, 1997, 1999; Xin and Wang, 2007; Huang et al., 2011). Huang et al. (2011) studied the effect of Ca²⁺ on the fermentation of polyglutamic acid. Addition of calcium chloride resulted in a 15.2% increase in final yield of polyglutamic acid, while the activities of enzymes involved in the metabolism of α-ketoglutaric acid were also improved (Huang et al., 2011). Ca²⁺ in the fermentation broth could also serve as an essential catalytic cofactor to activate the specific enzymes involved in certain metabolic pathways, resulting in redirection of the intracellular carbon and energy flux (Liu et al., 2007; Egnatchik et al., 2014).

Flux balance analysis (FBA), as one of the metabolic flux analysis (MFA) approaches, is to analyze the distribution of metabolic flux based on the quantitative equilibrium model of the metabolic network (also be called stoichiometric MFA) (Varma and Palsson, 1994; Papp and Simeonidis, 2007). The input data were mainly derived from the measurement of extracellular flux. The FBA has advantages over the traditional MFA of carbon labeling experiment (CLE) using the isotope labeling such as ¹³C (Ando and Garcia Martin, 2018), owing to the design flexibility and easiness of operation and analysis. As a popular tool for analyzing the metabolic flux, FBA methodology has been rapidly developed and the application has risen over the past more than 30 years, particularly with the purpose of calculation of the maximum theoretical output of metabolism (Krömer et al., 2006; Klamt et al., 2018; Rawls et al., 2019), description of the metabolic flux distribution of the complete central metabolism in industrial strains Corynebacterium glutamicum (Vallino and Stephanopoulos, 1993) and E. coli (Varma and Palsson, 1994), and estimation of the effects of genetic modification or environmental alteration on intracellular material or energy flux (Li et al., 2017; Zhao et al., 2017; Armingol et al., 2018; Hon et al., 2019; Martinez-Monge et al., 2019).

In this work, calcium was found to be capable of promoting L-Met biosynthesis in the genetically engineered E. coli W3110-BL. In order to deeply understand the role of calcium in L-Met metabolic pathway, the quantitative analysis of metabolic flux in the presence of calcium was conducted using FBA. The results were of much importance in developing molecular and engineering strategies for further modifying the L-Met biosynthetic pathway.

**Materials and Methods**

**Bacterial Strain and Plasmid**

The genetically engineered E. coli W3110-BL previously constructed in our laboratory (Huang et al., 2017, 2018) was used as a L-Met producing strain in this study.

**Media and Cultivation Conditions**

The seed medium was Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 10 g/L) supplemented...
### TABLE 1 | The metabolic reaction of the engineered E. coli W3110-BL.

| No. | Metabolic reaction |
|-----|--------------------|
| **Glycolysis** | |
| 1  | GLC + ATP → G6P + ADP |
| 2  | G6P ↔ F6P |
| 3  | F6P + ATP → GAP + ADP |
| 4  | GAP + ADP + NAD^+ ↔ 3PG + ATP + NADH |
| 5  | 3PG ↔ PEP |
| 6  | PEP + ADP → PYR + ATP |
| **Pentose phosphate pathway** | |
| 8  | G6P + 2 NADP^+ → R5P + 2 NADPH + CO_2 |
| 9  | R5P ↔ X5P |
| 10 | X5P ↔ G6P ↔ F6P + E4P |
| **TCA cycle** | |
| 7  | PYR + NAD^+→AcCoA + CO_2 + NADH |
| 26 | OXA + AcCoA ↔ CIT |
| 27 | CIT + NADP^+ ↔ α-KG + CO_2 + NADPH |
| 28 | α-KG + ADP + NAD^+ ↔ SUC + CO_2 + ATP + NADH |
| 29 | SUC + FAD^2+ ↔ FUM + FAH_2 |
| 30 | FUM + NAD^+ ↔ OXA + NADH |
| 42 | PEP + CO_2 → OXA |
| **Amino acid biosynthesis** | |
| 14 | 3PG + Glu + NAD^+ → Ser + α-KG + NADH |
| 15 | Ser + 2 NAD^+ → G1y + CO_2 + 2 NADH |
| 16 | Ser + SO_3^2- + 4 ATP + 4 NADPH → Cys + 4 ADP + 4 NADP^+ |
| 17 | E4P + 2 PEP + R5P + Glu + Ser + 3 ATP + NADPH → Trp + PYR + Glu + GAP + CO_2 + NADP^+ + 3 ADP |
| 18 | E4P + 2 PEP + Glu + ATP + NADPH → Phe + Gln + α-KG + CO_2 + ADP + NADP^+ |
| 19 | E4P + 2 PEP + Glu + ATP + NADPH + NAD^+ → Tyr + Gln + α-KG + CO_2 + ADP + NADP^+ + NADH |
| 22 | 2 PYR + AcCoA + Glu + NADPH + NAD^+ → Leu + α-KG + 2 CO_2 + NADH + NADP^+ |
| 23 | PYR + NH_4^+ + NADH → Ala + NAD^+ |
| 24 | 2 PYR + Glu + NADPH ↔ Val + α-KG + CO_2 + NADP^+ |
| 32 | α-KG + NH_4^+ + NADPH ? Glu + NADP^+ |
| 33 | Glu + NH_4^+ + ATP → Gln + ADP |
| 34 | OXA + Glu → Asp + α-KG |
| 35 | Asp + ATP + NADPH → Hom + NAD^+ |
| 36 | Hom + Cys → Met + PYR + α-KG + NH_4^+ + SUC |
| 37 | Hom + ATP → Thr + ADP |
| 38 | Thr + Glu + PYR + NADPH → Ile + α-KG + CO_2 + NH_4^+ + NADP^+ |
| **Transport reactions** | |
| 20 | PYR + CoA ↔ AcCoA + FOR |
| 21 | PYR + NADH ↔ NAD^+ + LAC |
| 25 | AcCoA + ADP ↔ ACE + ATP |
| **Oxidative phosphorylation/maintenance energy** | |
| 31 | FADH_2 + 1/2 O_2 + 2 ADP → 2 ATP + FAO_2^2+ |
| 40 | NADH + 1/2 O_2 + 3 ADP → 3 ATP + NAD^+ |
| 41 | ATP → ADP + Pi |
| **Biomass formation** | |
| 39 | 0.488 Ala + 0.229 Asp + 0.087 Cys + 0.250 Glu + 0.250 Gln + 0.582 Gly + 0.428 Leu + 0.146 Met + 0.176 Phe + 0.241 Thr + 0.054 Trp + 0.131 Tyr + 0.402 Val + 0.205 G6P + 0.071 F6P + 0.754 R5P + 0.129 GAP + 0.619 3PG + 0.276 ife + 0.051 PEP + 0.083 PYR + 2.510 AcCoA + 0.087 α-KG + 0.340 OXA + 33.247 ATP + 5.363 NADPH → Biomass + 1.455 NADH |

Biomass: C_{1.18}H_{1.8}O_{0.6}N_{0.2}
with 100 µg/mL Amp. The fermentation medium contained 20 g/L glucose, 5 g/L (NH$_4$)$_2$SO$_4$, 1.5 g/L yeast extract, 2 g/L KH$_2$PO$_4$, 10 g/L Na$_2$S$_2$O$_3$, 1 mg/L Vb$_{12}$, 1 mL/L salt solution (MgSO$_4$·7H$_2$O 0.5 g/L, MnSO$_4$·8H$_2$O 5 mg/L, FeSO$_4$·7H$_2$O 5 mg/L, ZnSO$_4$ 5 mg/L), 50 mg/L L-lysine, 100 µg/mL Amp, and 0.1 mmol/L isopropyl β-d-1-thiogalactopyranoside (IPTG). Different amounts of calcium carbonate (CaCO$_3$) were supplemented when required.

In batch cultivation for L-Met production, 150 mL of the seed broth after cultivation in LB medium containing 100 µg/L ampicillin for 12 h at 37°C, 180 rpm was inoculated into a 5-L jar fermenter (Winpact Parallel Fermentation System FS-KH, 1.5 vvm). 100 mg/mL Amp was supplemented when required. IPTG was added at the final concentration of 0.1 mmol/L and the inducible expression was conducted at 28°C. During the entire fermentation process, the dissolved oxygen (DO) level was maintained above 20% by controlling the agitation rate at 300–500 rpm (Supplementary Figure S1).

**Construction of Metabolic Network of the Engineered E. coli W3110-BL**

As a workhorse microorganism in biomanufacturing, E. coli has been extensively used for the biotechnological production of amino acids. Herein, the strain E. coli W3110-BL developed in our previous work by means of genetic manipulations was used for L-Met production.

### TABLE 2 | The metabolites of E. coli W3110-BL and the reaction equations.

| No. | Metabolites | Equation |
|-----|-------------|----------|
| 1   | ATP         | $r_1 - r_3 + r_4 + r_6 - 4r_16 - 3r_17 - r_18 - r_19 + r_25 + r_28 + 2r_31 - r_33 - r_35 - r_37 - 33.247 r_39 + 3r_40 - r_41$ |
| 2   | NADH        | $r_4 + r_7 + r_{14} + 2r_{15} + r_{19} - r_{21} + r_{22} - r_{23} + r_{28} + r_{30} + 1.545 r_39 - r_40$ |
| 3   | NADPH       | $2r_8 - 4r_{16} - r_{17} - r_{18} - r_{19} - r_{22} - r_{24} + r_7 - r_{32} - r_{35} - r_{38} - 5.363 r_39$ |
| 4   | FADH$_2$    | $r_29 - r_31$ |
| 5   | G6P         | $r_1 - r_2 - r_8 - 0.025 r_39$ |
| 6   | F6P         | $r_2 - r_3 + r_{12} + r_{13} - 0.071 r_39$ |
| 7   | 3PG         | $r_4 - r_5 - r_{14} - 0.619 r_39$ |
| 8   | PEP         | $r_5 - r_6 - 2r_{17} - 2r_{18} - 2r_{19} - 0.051 r_39 - r_42$ |
| 9   | PYR         | $r_6 - r_7 + r_{17} - r_{20} - r_{21} - 2r_{22} - r_{23} - 2r_{24} - r_{38} - 0.803 r_39$ |
| 10  | AcCoA       | $r_7 + r_{20} - r_{22} - r_{25} - r_{26} - 2.510 r_39$ |
| 11  | CIT         | $r_{26} - r_{27}$ |
| 12  | RIL5P       | $r_8 - r_9 - r_{10}$ |
| 13  | X5P         | $r_9 - r_{11} - r_{13}$ |
| 14  | R5P         | $r_{10} - r_{11} - r_{17} - 0.754 r_39$ |
| 15  | S7P         | $r_{11} - r_{12}$ |
| 16  | GAP         | $2r_3 - r_4 + r_{11} - r_{12} + r_{13} + r_{17} - 0.129 r_39$ |
| 17  | E4P         | $r_{12} - r_{13} - r_{17} - r_{18} - r_{19}$ |
| 18  | α-KG        | $r_{14} + r_{18} + r_{19} + r_{22} + r_{24} + r_{27} - r_{28} - r_{32} + r_{34} + r_{38} - 0.087 r_39$ |
| 19  | SUC         | $r_{28} - r_{29} + r_{36}$ |
| 20  | FUM         | $r_{29} - r_{30}$ |
| 21  | OXA         | $r_{26} + r_{30} - r_{34} - 0.34 r_39 + r_42$ |
| 22  | Asp         | $r_{34} - r_{35} - 0.229 r_39$ |
| 23  | Hom         | $r_{35} - r_{36} - r_{37}$ |
| 24  | Thr         | $r_{37} - r_{38} - 0.241 r_39$ |
| 25  | G1u         | $r_{14} + r_{17} - r_{18} - r_{19} - r_{24} + r_{32} - r_{33} - r_{34} - 0.25 r_39$ |
| 26  | Ala         | $r_{23} - 0.488 r_39$ |
| 27  | Cys         | $r_{16} - 0.087 r_39$ |
| 28  | Gin         | $r_{17} + r_{18} + r_{19} - r_{22} + r_{33} - 0.25 r_39$ |
| 29  | Gly         | $r_{15} - 0.582 r_39$ |
| 30  | Leu         | $r_{22} - 0.428 r_39$ |
| 31  | Met         | $r_{36} - 0.146 r_39$ |
| 32  | Phe         | $r_{18} - 0.176 r_39$ |
| 33  | Tyr         | $r_{19} - 0.131 r_39$ |
| 34  | Val         | $r_{24} - 0.402 r_39$ |
| 35  | Ser         | $r_{14} - r_{15} - r_{16} - r_{17}$ |
| 36  | Ile         | $r_{38} - 0.276 r_39$ |
The metabolic network of the *E. coli* W3110-BL with glucose as sole carbon source was constructed based on the KEGG metabolic pathway database and the other publications (Koffas et al., 1999; Hong and Lee, 2001), which consisted of 42 reactions and 36 metabolic intermediates (Tables 1, 2; the abbreviation of each metabolite was shown in Supplementary Table S2). As shown in Figure 1, with glucose as carbon source, the carbon flux passed through a series of metabolic pathway or biochemical reactions, mainly including Embden-Meyerhof-Parnas pathway (EMP pathway), pentose phosphate pathway (PP pathway), tricarboxylic acid cycle (TCA cycle), transport reactions, oxidative phosphorylation, biomass formation, and L-Met biosynthesis. For the ease of calculation and analysis, part of the metabolic pathways were simplified according to the reported approaches (He et al., 2014): (1) the amino acid degradation reaction, the nucleotide salvage pathway, and other carbon source utilization pathways were omitted; (2) The routes of other amino acids metabolism were not included, except for the essential biosynthesis pathway of the amino acids detectable in the fermentation broth; (3) The cell composition (biomass) was simplified to consist of proteins, DNA, RNA, fatty acids, and cell walls; and the synthesis of fatty acids and cell walls was expressed

**FIGURE 1** | The central metabolism network of *E. coli* W3110-BL. Abbreviations: G6P, glucose-6-phosphate; F6P, 6-phosphate fructose; GAP, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYK, pyruvate; AcCoA, acetyl coenzyme A; α-KG, α-ketoglutarate; RL5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, sedoheptulose-7-phosphate.
as the precursors synthesis in the metabolic network; (4) Since the branch pathway for L-lysine formation had been knocked out during the construction process of the engineered strain (Huang et al., 2017), the synthetic route of L-lysine was not considered in this study.

**Theory of FBA**

Metabolic networks are non-linear complex networks regulated by kinetic mechanisms, usually involving hundreds of metabolites and thousands of reactions. Constructing genome-scale metabolic models manually is time-consuming and labor-intensive. Therefore, it is necessary to build mathematical models of these metabolic networks to describe, understand, and eventually predict the system metabolic behavior (Tran et al., 2008). Generally, the metabolic network is constructed by aggregating the reported data in various literature and databases. After construction of the metabolic network, the flux distribution of the metabolic network is to be determined.

In order to elucidate the reasons for the improvement of L-Met production by addition of CaCO$_3$, a metrological model FBA, based on mass conservation and pseudo-steady-state (PSS) of metabolic intermediates (Stephanopoulos and Sinskey, 1993), was adopted to estimate the effects of CaCO$_3$ on metabolic flux distribution of the *E. coli* W3110-BL. First, assuming that the net change in the concentration of either reactants or products in the metabolic network was 0, and the reaction was in a state of equilibrium. There were $n$ constraints on reaction rates change derived from $n$ intermediates. Then assuming the total number of rates needed to be determined is $J$, so the degree of freedom was $F = J - n$.

The flux distribution of the complete metabolic network can be determined by measuring the extracellular metabolites only if their number is more than the degree $F$. For each metabolite, the mass balance can be expressed as:

$$\frac{dX_i}{dt} = \sum_j S_{ij}V_j$$

where, $S_{ij}$ in the metrology matrix represents the number of moles of metabolite $i$ that produced or consumed in reaction $j$. $V_j$ is the $j$th flux in the system; $X_i$ stood for the concentration of metabolite.

When the metabolic network is at steady state, according to the principle of mass balance, the total inputting flux of each metabolite in the network toward its metabolic pool is equal to the output, so the formula could be reduced to a set of homogeneous linear equation, described in matrix notation as:

![Figure 2](https://example.com/fig2.png)

**FIGURE 2** | Time course of fermentative production of L-Met with *E. coli* W3110-BL in 5-L fermentor supplemented with different amounts of CaCO$_3$. (A) glucose consumption; (B) cell growth; (C) L-Met biosynthesis; (D) pH variation. All measurements were performed in triplicate. For clarity, the error bars were not displayed.
\[ S \cdot v = 0 \]  

(2)

Where, \( S \) was an \( m \times n \) metrology matrix. \( m \) was the number of metabolites and \( n \) the number of reactions in the system. \( v \) was an \( m \)-dimensional metabolic reaction rate vector.

In this study, the constructed model included 42 metabolic reactions and 36 metabolites (Figure 1). The freedom degree of the matrix was six. The specific cell growth rate \( r_{39} \), specific glucose consumption rate \( r_{1} \), as well as the specific accumulation rates of formic acid \( r_{20} \), lactic acid \( r_{21} \), acetic acid \( r_{25} \), succinic acid \( r_{28} \), and L-Met \( r_{36} \), were determined based on the extracellular measurement. The positive definite matrix was calculated by Matlab 2016a software (Morales et al., 2016). All fluxes were converted to percentages based on a glucose uptake rate of 100 mmol/g cell/h.

**Analytical Methods**

The extracellular concentrations of pyruvic acid, formic acid, lactic acid, acetic acid, \( \alpha \)-ketoglutarate, and succinic acid were determined by high-performance liquid chromatography (HPLC) (Waters, Milford, MA, United States) equipped with a RID detector (Waters 2410, Waters, Milford, MA, United States). For metabolites separation, an Aminex HPX-87H column (BioRad, München, Germany) was used, working at 35°C and a flow rate of 0.6 mL/min with 5 mmol/L sulfuric acid as mobile phase. Biomass, residual glucose, and L-Met was measured as described previously (Zhou et al., 2019). The specific oxygen uptake rate was measured by the dynamic method (Santos et al., 2006). The specific carbon dioxide production rate was determined by a titrimetric method (Conventi et al., 2003).

For preparation of cell-free extracts, the *E. coli* cell pellets were collected, washed and suspended in 100 mM potassium phosphate buffer (pH 7.0). The pellets were disrupted by sonication (work time 5 s, interval 10 s, total time 2 min) in an ice bath using an ultrasonic processor VCX 130PB (Sonic & Materials Inc., Newtown, United States). Cellular debris was removed by centrifugation at 12,000 g and 4°C for 5 min. The crude extracts were used for assays of enzyme activity and intracellular calcium ions level. Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

A Citrate Synthase Activity Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used for Citrate synthase (CS) activity detection. According to the instruction of Kit, CS activity was assayed *spectrophotometrically* at 412 nm using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) colorimetric assay method (Srere, 1969; Quandt et al., 2015).

The calcium concentration was assayed using a Calcium Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China)
with methylthymol blue (MTB) as a color agent (Themelis et al., 1999). The absorbance of the colored Ca-MTB complex produced was monitored at 610 nm by a Microplate Spectrophotometer SpectraMax M2 (Molecular Devices, CA, United States).

The transcriptional responses of the genes associated with the TCA cycle to CaCO$_3$ addition were detected by the qRT-PCR. Samples were taken at the logarithmic growth phase (15 h) during the fermentation process with or without addition of CaCO$_3$ and treated by flash-freezing in liquid nitrogen. The total RNA was extracted using RNAiso Plus reagent (Takara, Kyoto, Japan) according to the manufacturer’s instructions. The RNA quality was determined using 1% agarose gel electrophoresis and the quantity was measured with a NanoDrop 2000c UV–vis Spectrophotometer (Thermo Scientific, Madison, United States). Each RNA sample was reverse-transcribed to cDNA by the PrimeScript™ RT reagent Kit (Takara, Kyoto, Japan) according to the manufacturer’s manual. The qRT-PCR was carried out on a LightCycler 480 System (Roche, Basel, Switzerland) using TB Green real-time PCR mix (Takara, Kyoto, Japan) with the specific primers (Supplementary Table S1). The house-keeping gene 16S rRNA was used as an internal control. The PCR amplification was performed in 10 µL reaction mixtures, which consisted of 3 µL 2 × SYBR Premix Ex Taq, 0.5 µL diluted cDNA, 0.2 µL forward and reverse primer, and 4.1 µL RNase free water. All the PCR reactions were carried out using the following protocol: 95°C for 30 s; 40 cycles of 94°C for 3 s and 60°C for 30 s. The qRT-PCR for each sample was performed in triplicate and the results were analyzed with the 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

**Statistical Analysis**

All experiments were carried out in triplicate and the average values were reported. The values of average and standard deviation (SD) were calculated in Microsoft Excel. The statistical differences were determined according to student’s t-test. P values of <0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**CaCO$_3$ Addition Improves L-Met Biosynthesis in the Engineered E. coli W3110-BL**

For L-Met biosynthesis, the engineered E. coli W3110-BL was grown in batch cultures performed in 5-L fermentor, during which the pH of the fermentation medium was maintained at 6.8 ± 0.2 either with 30% NH$_3$-H$_2$O or with CaCO$_3$ in different concentrations (10, 20, 30, and 40 g/L). As shown in Figure 2, when the pH was adjusted with 30% NH$_3$-H$_2$O, only 0.94 g/L L-Met was accumulated; while with CaCO$_3$ as pH regulator, L-Met accumulation was enhanced compared with that of the control (with 30% NH$_3$-H$_2$O). Furthermore, L-Met titer was increased as the CaCO$_3$ amount raised from 0 to 30 g/L, with the maximum value of 1.48 g/L at 30 g/L CaCO$_3$ (57.45% higher than that of the control). The yield of L-Met against glucose was 0.089 mol/mol, considerably increased compared with that of the control (0.057 mol/mol). However, with CaCO$_3$ further increased to 40 g/L, decreased L-Met production was observed. Therefore, from the view of fermentation optimization, 30 g/L CaCO$_3$ was determined to be most conducive to L-Met biosynthesis.

It should be noted that, when using CaCO$_3$ as pH regulator, the pH of the fermentation medium was not constant. As was seen from Figure 2D, in batch cultivation with different amounts of CaCO$_3$ addition, the pH was approximately at 6.8 (set value) in the initial stage and slowly varied (first decreased and then increased) between 5.5 and 8.2 with different patterns till the end of the fermentation.

To clarify whether pH or the presence of calcium really affected the L-Met production, other two different modes of batch fermentation were also tried. In Mode I, the pH was maintained at 6.8 ± 0.2 by automatic addition of 30% NH$_3$-H$_2$O as well as supplement of 30 g/L CaCO$_3$; in Mode II, CaCl$_2$ (33.3 g/L) was used to displace CaCO$_3$. Herein, the mode with pH controlled at 6.8 ± 0.2 by automatic addition of 30% NH$_3$-H$_2$O was named as Control-I, and the one only with 30 g/L CaCO$_3$ as pH regulator was named as Control-II. Except for the pH in Control-II that was varied between 5.5 and 8.2 over time (Figure 2D), the pH values in Control-I, Mode I, and Mode II were all well controlled at about 6.8 (data not shown).

As shown in Figure 3, with different modes of pH control, the profiles of glucose consumption and cell growth exhibited no crucial distinction among all the batch runs. Interestingly, when the pH was maintained at 6.8 ± 0.2 by 30% NH$_3$-H$_2$O, either with addition of CaCO$_3$ (Mode I) or CaCl$_2$ (Mode II), the L-Met accumulation was also greatly improved, 59.57 and 58.51% higher than that of the Control-I, respectively.

The fermentation patterns in Control-II and Mode I which both contained 30 g/L CaCO$_3$ were compared and analyzed further. Interestingly, although the extracellular pH values of E. coli cells in these two cases were different, there was no significant difference with respect to L-Met biosynthesis. This
indicated that calcium ions rather than environmental pH were most responsible for the enhancement of L-Met accumulation.

As shown in Figure 4, the intracellular calcium concentrations in Control-II and Mode II were in the same order of magnitude, although the solubility of CaCO₃ is much lower than that of CaCl₂ in aqueous solution. And it was considerably higher in each mode (1–10 mmol/L) in comparison with that in Control-I (0.5–8.0 µmol/L), implying that the consumption of calcium ions (intracellular free Ca²⁺ concentration) was highly correlated with the accumulation of L-Met.

**By-Products Formation in the Batch Fermentation With CaCO₃ Addition**

The formed by-products were detected and the results were illustrated in Figure 5. With the existence of CaCO₃, the

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**FIGURE 5** | Biosynthesis of organic acids in batch cultures in 5-L fermentor without or with CaCO₃ (30 g/L) addition. SD is shown for triplicate measurements of each organic acid. (A) α-KG, (B) succinic acid, (C) lactic acid, (D) formic acid, and (E) acetic acid.
maximum concentrations of main organic acids including \(\alpha\)-ketoglutarate, succinic acid, lactic acid, formic acid, and acetic acid were increased to different degrees. As for \(\alpha\)-ketoglutarate and succinic acid, the highest accumulation in the case of CaCO\(_3\) addition was increased by 50 and 20.3%, respectively, compared with that of the control. It was speculated that the presence of CaCO\(_3\) probably increased the carbon flux of the TCA cycle. After the glucose was exhausted, the accumulations of \(\alpha\)-ketoglutarate and succinic acid were both decreased, likely due to their conversion to other metabolic products. By contrast, the biosynthesis of formic acid, lactic acid, and acetic acid was not immediately influenced by glucose limitation in the presence of CaCO\(_3\). All the main organic acids formed in the fermentation process might be re-utilized for maintenance of cell growth or \(\text{L-Met}\) biosynthesis in the late fermentation phase.

The Effect of CaCO\(_3\) Addition on Carbon Metabolic Flux Distribution

In order to reveal the specific role of CaCO\(_3\) addition on the \(\text{L-Met}\) biosynthesis pathway in \(E.\ coli\) W3110-BL, the intracellular metabolic flux distribution was estimated using the FBA method. 12 reaction rates including the specific consumption rate of glucose (r1)/oxygen and the specific formation rate of formic acid (r20), lactic acid (r21), \(\alpha\)-ketoglutarate, succinic acid (r28), \(\text{L-Met}\) (r36), biomass (r39), carbon dioxide, pyruvic acid, and other amino acids (L-isoleucine, L-leucine, L-threonine, L-homoserine, and L-tyrosine) were calculated and summarized in Table 3. The other amino acids were expressed as a homogenized chemical with a formula of C\(_5\)H\(_{11}\)NO\(_2\)\(_6\).

Carbon recoveries as biomass and various metabolites were 97.4–102.1% of the theoretical maximum, suggesting that the major metabolites were properly identified and quantified. The reduction degree calculated based on the equation \(\varepsilon = 4\ C + H – 2\ O – 3\ N\) (Oh et al., 2008) were well balanced. Among these reaction rates, r1, r20, r21, r25, r28, r36, and r39 were used to determine the volumetric formation rates of intracellular metabolites.

The metabolic model included the main biochemical pathways in carbon central metabolism: glycolysis, PP pathway, TCA cycle, amino acid synthesis, transport reactions, oxidative phosphorylation, and biomass formation. The average intracellular metabolic flux distribution in the late logarithmic growth phase (19 h) was calculated and shown in Figure 6. This model developed herein was simple but biologically meaningful in description of the central metabolism and \(\text{L-Met}\) biosynthesis, thus the quality of the outcome computed from the model was valid in estimating the effect of CaCO\(_3\) addition on \(E.\ coli\) intracellular flux distributions in batch cultures.

As shown in Figure 6, carbon flux in the central metabolism was redistributed in response to CaCO\(_3\) addition. The carbon flux into \(\text{L-Met}\) biosynthesis was increased by 44.83% in the case of CaCO\(_3\) addition, whereas the carbon flux into the TCA cycle was also increased considerably. Under the aerobic condition, the glycolysis and TCA cycle were correlated by the conversion of PEP to oxaloacetate catalyzed by phosphoenolpyruvate carboxylase as well as the formation of citrate from oxaloacetate and acetyl-CoA catalyzed by the citrate synthase. It could be seen from Figure 6 that the carbon flux into oxaloacetate from PEP was slightly varied, while the flux for citrate biosynthesis from oxaloacetate and acetyl-CoA was increased by 46.67%. Therefore, the increase of carbon flux in the TCA pathway was probably achieved by directing the metabolites flux into citrate biosynthesis from the pool of PEP, pyruvate, and the acetyl-CoA.

With CaCO\(_3\) addition, the relative changes in transcription levels of 15 genes involved in TCA cycle were determined by qRT-PCR. As shown in Figure 7, the transcriptional level of 7 genes including \(\text{gltA}\) encoding CS, \(\text{acnA}\) encoding aconitase (ACO) isoenzyme, \(\text{idcA}\) encoding isocitrate dehydrogenase

| TABLE 3 | Rate parameters of metabolic reaction in \(E.\ coli\) W3110-BL. |
|------------------|------------------|------------------|------------------|
| Fermentation parameters | Chemical formula | Control | CaCO\(_3\) addition |
| **Specific consumption rate (mmol/g DCW/h)** | | | |
| Glucose (r1) | C\(_6\)H\(_{12}\)O\(_6\) | 0.96 ± 0.056 | 0.77 ± 0.022 |
| Oxygen | O\(_2\) | 1.330 ± 0.098 | 1.08 ± 0.045 |
| **Specific formation rate (mmol/g DCW/h)** | | | |
| Formic acid (r20) | CH\(_2\)O | 0.41 ± 0.017 | 0.33 ± 0.020 |
| Lactic acid (r21) | C\(_3\)H\(_6\)O\(_3\) | 0.10 ± 0.007 | 0.04 ± 0.003 |
| Acetic acid (r25) | C\(_2\)H\(_4\)O\(_2\) | 0.32 ± 0.010 | 0.37 ± 0.018 |
| \(\alpha\)-Ketoglutarate | C\(_5\)H\(_4\)O\(_5\) | 0.02 ± 0.001 | 0.09 ± 0.004 |
| Succinic acid (r28) | C\(_4\)H\(_6\)O\(_4\) | 0.14 ± 0.010 | 0.19 ± 0.002 |
| \(\text{L-Met}\) (r36) | C\(_6\)H\(_{11}\)O\(_2\)NS | 0.06 ± 0.003 | 0.07 ± 0.004 |
| Cell growth (h\(^{-1}\)) (r39) | C\(_6\)H\(_{10}\)O\(_2\)N\(_2\) | 0.03 ± 0.002 | 0.02 ± 0.004 |
| Carbon dioxide | CO\(_2\) | 1.25 ± 0.063 | 0.98 ± 0.033 |
| Pyruvic acid | C\(_3\)H\(_4\)O\(_3\) | 0.11 ± 0.010 | 0.02 ± 0.003 |
| Other amino acids\(^a\) | C\(_5\)H\(_{11}\)NO\(_2\)\(_6\) | 0.08 ± 0.002 | 0.02 ± 0.001 |
| **Carbon balance (%)** | 97.4 ± 1.83 | 102.1 ± 2.44 |
| **Reduction degree (%)** | 94.4 ± 3.60 | 99.0 ± 3.29 |

\(^{a}\)Other amino acids included L-isoleucine, L-leucine, L-threonine, L-homoserine, and L-tyrosine. For convenience, these amino acids were expressed as a homogenized chemical with a formula of C\(_5\)H\(_{11}\)NO\(_2\)\(_6\).
(ICDH), *sucC* encoding succinyl-CoA synthetase (SCS) isoenzyme, *sdhC/sdhD* encoding succinate dehydrogenase (SDH) isoenzymes, and *fumB* encoding fumarase (FH) isoenzyme were higher than those of the control (without CaCO$_3$ addition), indicating an up-regulated expression of these genes. By contrast, the expressions of 6 genes including *acnB* encoding ACO isoenzyme, *sucB* encoding α-ketoglutarate dehydrogenase (KGDH) isoenzyme, *sdhA/sdhB* encoding SDH isoenzymes, *fumA* encoding FH isoenzyme, and *mdh* encoding malate dehydrogenase (MDH) were down-regulated. While, there were no significant changes in the relative transcriptional quantitation of *sucA* encoding KGDH isoenzyme and *sucD* encoding SCS isoenzyme. Although the transcriptional responses of these 15 genes to CaCO$_3$ supplement were different, it could be certain that, the expression of CS, ICDH, and SCS were enhanced due to the presence of CaCO$_3$.

To evaluate the effect of Ca$^{2+}$ on intracellular CS activity during L-Met biosynthesis, CS specific activity was monitored in cell-free crude extract by means of DTNB colorimetric assay method. As illustrated in Figure 8, the activation of CS by Ca$^{2+}$ before 15 h was not observed. However, the specific activity of CS was much higher than that of the control after 15 h, especially during 22–48 h. The increase in CS activity was allowed for perturbation of the acetyl-CoA branch point with enhanced TCA influx in the presence of CaCO$_3$.

The results indicated that, CaCO$_3$ addition could direct more carbon to participate in L-Met biosynthesis, through fluctuation of the carbon balance in some essential metabolic pathways, particularly in the TCA cycle. CS, responsible for the entry reaction of a two-carbon unit into the TCA cycle, was thus considered to be a promising metabolic control node for L-Met overproduction.

**The Effect of CaCO$_3$ Addition on ATP and Cofactor Metabolism**

The formation and consumption of ATP, NADH, NADPH, and FADH$_2$ in the related biochemical pathways was estimated.
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**FIGURE 7** | The transcriptional responses of the genes involved in TCA cycle to CaCO$_3$ addition. The dashed line represented the relative fold change of gene expression of in the control (the value is 1.0).

**FIGURE 8** | Specific activities of citrate synthase (CS) of E. coli W3110-BL during growth in the batch cultivation with or without CaCO$_3$ addition. The CS activity was measured in crude cell extracts and all measurements were performed in triplicate.

**FIGURE 9** | Estimated production and consumption of ATP, NADH, NADPH, and FADH$_2$ by the E. coli W3110-BL without or with CaCO$_3$ addition. All data represent the average value of three estimation. For clarity, the error bars were not shown. The hydrolysis of ATP to ADP was not included in this figure.

and presented in Figure 9. ATP was mainly produced through glycolysis, TCA cycle, transport reactions pathways, and oxidative phosphorylation; and it was consumed by amino acid synthesis and biomass formation. The ATP formation in the presence of CaCO$_3$ was obviously improved compared with the condition of CaCO$_3$ absence. ATP was generated at a relative high level, particularly in the oxidative phosphorylation pathway. Therefore, CaCO$_3$ addition could provide more energy for L-Met biosynthesis.

The major pathway of NADPH supply included PP pathway and TCA cycle, and NADPH was mainly consumed by amino acid synthesis and biomass formation. Different from NADPH, NADH was synthesized through glycolysis and TCA cycle, and oxidative phosphorylation was its dominant consumption pathway. The main source of FADH$_2$ was TCA cycle and it was solely used for oxidative phosphorylation. These results were in good agreement with those of other studies (Kind et al., 2013; He et al., 2014). As Figure 9 indicated, Ca$^{2+}$ could regulate the intracellular cofactor levels, through increasing the generation of NADPH, NADH and FADH$_2$ in TCA cycle, or increasing the consumption of NADH and FADH$_2$ in oxidative phosphorylation.

NADH, FADH$_2$, and NADPH are the key cofactors (or coenzymes) in the cellular metabolic network. NADH and FADH$_2$ function as electron carriers and participate in almost all oxidation-reduction reactions. They play crucial roles in cellular energy generation in the living cells. Generally, the ATP yielded during direct glucose metabolism is very limited, while both NADH and FADH$_2$ involved in the process of cellular respiration can help in producing more ATP through electron transfer and oxidative phosphorylation reaction. NADPH usually acts as a hydrogen donor to participate in various metabolic reactions (Kind et al., 2013); Besides, it affects numerous enzymatic activities and endogenous concentrations of regulators in many chemical reactions (Xu et al., 2018), such as the synthesis pathway of lipids, fatty acids, and nucleotides. Therefore, the level of cofactors NADH, FADH$_2$, and NADPH are essential to amino acid biosynthesis via microbial fermentation (Singh et al., 2007). It has been proved that, increasing the supply of NADPH has a positive effect on the fermentative production of amino acids (Sharp, 2013; Wang et al., 2013). Furthermore, based on
regulation of the intracellular cofactor level, optimization of cellular physiological and metabolic functions to maximize the carbon flux distribution and energy supply for the biosynthetic pathway of several kinds of amino acids has been achieved (Bartek et al., 2010; Yin et al., 2014; Xu et al., 2018).

As a summary, exogenous addition of CaCO_{3} could regulate the oxidative phosphorylation pathway to produce more ATP. Additionally, the intracellular cofactors concentration also varied due to CaCO_{3} existence. These results suggested that the improvement of L-Met biosynthesis was correlated with ATP and cofactor metabolism, and was likely due to the collaborative contribution of these effects. It provided an important reference in metabolic engineering to channel more energy flux into the synthesis of target amino acid.

CONCLUSION

The positive impact of Ca^{2+} on L-Met biosynthesis of the engineered E. coli W3110-BL was demonstrated through comparative assay of different modes of batch fermentation with or without Ca^{2+} addition into the fermentation medium. Intracellular metabolic flux distribution and the metabolism of ATP and cofactors in response to Ca^{2+} existence at the late logarithmic growth phase (19 h) were estimated by FBA. The results indicated that addition of CaCO_{3} (30 g/L) into the fermentation medium resulted in an increased metabolic flux in TCA cycle and a relatively high level of ATP and cofactors generation, which may collaboratively contribute to the enhancement of L-Met biosynthesis. This study lay important fundament for further modification of the potential metabolic control node to channel more metabolic flux into L-Met over-production.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

H-YZ, W-JW, KN, Z-QL, and Y-GZ participated in designing of work. The analysis and interpretation of experimental the data was jointly conducted by all authors. H-YZ and W-JW prepared the manuscript with the help of Z-QL. W-JW, Y-YX, and BZ performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00300/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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