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Optimization of L-Citrulline Operon in *Corynebacterium glutamicum* for L-Citrulline Production

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Abstract: L-citrulline plays important roles in many physiological processes, and its application range is expanding rapidly. *Corynebacterium glutamicum* strains have the potential to be efficient L-citrulline producers. In this study, we performed optimization of L-citrulline biosynthesis operon in *C. glutamicum* ATCC13032 for L-citrulline production. Chromosomal integration of the integral $argB_{Ec}$ gene from *Escherichia coli* (encoding natively insensitive *N*-acetylglutamate kinase), the deletion of the *argR* gene (encoding repressor ArgR), and the deletion of the *argG* gene (encoding argininosuccinate synthase) were achieved simultaneously by one-step genome modification and by obtaining the L-citrulline-producing strain. Then, plasmid-based overexpression of the optimized L-citrulline operon was carried out and the L-citrulline production was further improved. In fed-batch fermentation, the L-citrulline production and yield from glucose of the final strain reached 26.7 g/L and 0.18 g/g, respectively. These results indicate that optimization of L-citrulline operon in *C. glutamicum* is effective to construct the L-citrulline over-producing strain.

Keywords: *Corynebacterium glutamicum*; L-citrulline; *N*-acetylglutamate kinase; ArgR repressor; argininosuccinate synthase

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1. Introduction

L-citrulline plays important roles in many physiological processes, such as the regulation of nitrogen homeostasis, immune-system stimulation, the antioxidant effect, and nitric oxide metabolism. Due to its multiple functions, L-citrulline is widely used in the pharmaceutical, health-care food, and cosmetics industries, and its application range is expanding rapidly [1–3].

In recent years, methods for L-citrulline production by enzymatic synthesis and microbial fermentation have been reported. The enzymatic synthesis uses arginine deiminase for the hydrolysis of the arginine guanidinium group [2,3]. In the enzymatic method for L-citrulline production, L-arginine is the substrate or raw material. However, L-arginine is produced by microbial fermentation at present, and the L-arginine production strains are ordinarily derived from *Corynebacterium glutamicum* and its related species [4–6]. In *C. glutamicum* strains, L-citrulline is an intermediate metabolite in the L-arginine biosynthetic pathway (Figure 1). During L-arginine production by the microbial fermentation process, in the cells of the L-arginine producing strain, L-arginine is synthesized from L-citrulline by introducing the imino group in the carbamoyl of L-citrulline [4]. However, in an enzymatic method for L-citrulline production, the introduced imino group in L-arginine is hydrolyzed. Therefore, in consideration of the acquisition of the L-arginine substrate, the enzymatic method for L-citrulline production is uneconomical. In comparison, the one-step production of L-citrulline by microbial fermentation is more economical.

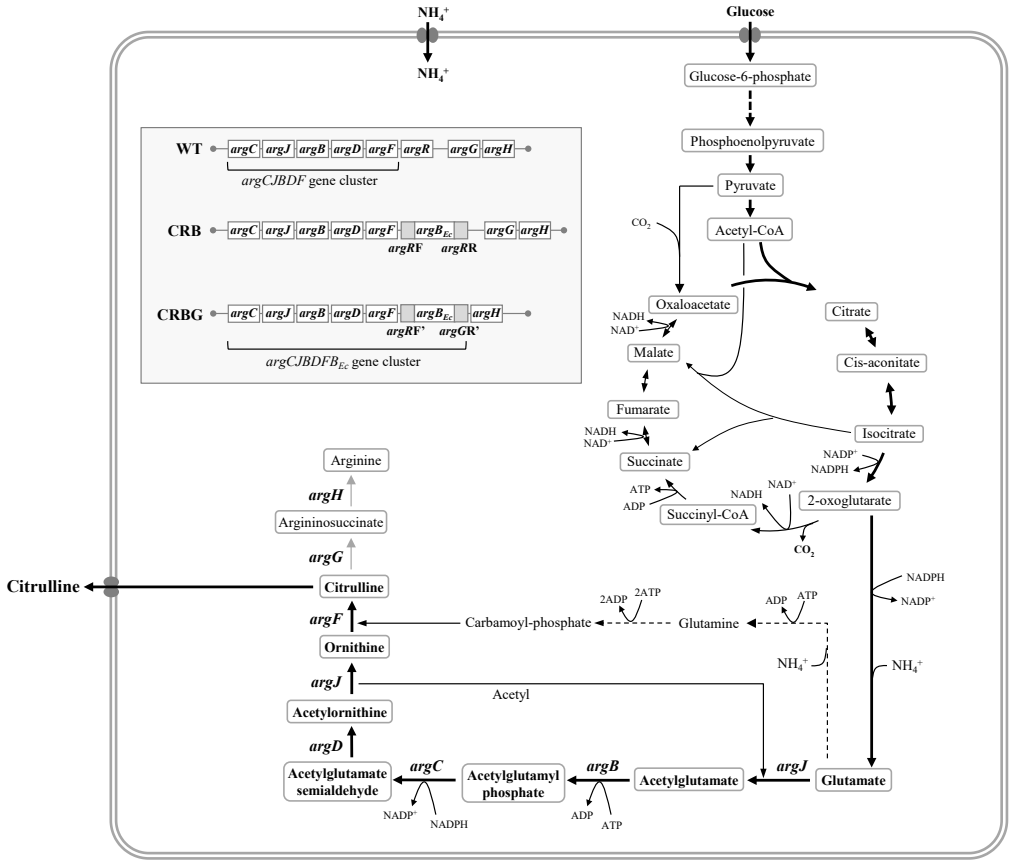

Figure 1. Schematic representation of the L-citrulline biosynthesis pathway in *C. glutamicum* and the modifications of the genome.

C. glutamicum and its related species have been employed for the industrial production of amino acids for several decades, and it is especially known for its ability to synthesis and secrete glutamate [7–10]. On this basis, *C. glutamicum* is considered an excellent host for the production of glutamate-derived amino acids, such as L-ornithine and L-arginine [9,10]. The genome of *C. glutamicum* reveals a rich repertoire of pathways, suggesting attractive properties for industrial production purposes; moreover, a wide range of genetic engineering tools for *C. glutamicum* engineering are established, and the omics data of *C. glutamicum* are constantly expanding. Therefore, metabolic engineering on *C. glutamicum* is likely to obtain a promising cell factory [7,9,10]. As mentioned above, in *C. glutamicum* strains, L-citrulline is an intermediate metabolite in the L-arginine biosynthetic pathway (Figure 1). In *C. glutamicum*, the genes involved in L-arginine biosynthesis are organized as the gene cluster *argCJBDFRGH* and are divided into *argCJBDFR* and *argGH* operons [5,11,12]. Additionally, the *argCJBDFR* operon encodes all of the enzymes required to convert L-glutamate to L-citrulline (Figure 1). The *argR* gene encodes repressor ArgR of the *argCJBDFR* and *argGH* operons. The *argG* gene encodes argininosuccinate synthase, which catalyzes the conversion of L-citrulline to argininosuccinate (Figure 1). The *argB* gene encodes *N*-acetylglutamate kinase (NAGK), which is feedback-inhibited by L-arginine, and the intracellular activity of NAGK is the limitation for L-arginine and L-citrulline synthesis by *C. glutamicum* [4,13,14]. However, in *Escherichia coli*, the $NAGK_{Ec}$ encoded by $argB_{Ec}$ is natively insensitive to L-arginine. In *C. glutamicum*, the replacement of the chromosomal *argB* by $argB_{Ec}$ could effectively improve L-arginine and L-citrulline production [13]. In *C. glutamicum*, introducing mutant NAGK that was less sensitive to L-arginine was also beneficial for L-ornithine production [8,15].

In *C. glutamicum* strains, L-citrulline is an intermediate metabolite between L-ornithine and L-arginine in the L-arginine biosynthetic pathway (Figure 1). Metabolically engineered

C. glutamicum could produce 51.5 g/L of L-ornithine with the productivity of 1.29 g/L/h in fed-batch fermentation [15]. Systems metabolic engineering in combination with random mutagenesis on *C. glutamicum* could result in the efficient production of L-arginine to 92.5 g/L in fed-batch fermentation [5]. Therefore, *C. glutamicum* strains have the potential to be efficient L-citrulline producers. The inactivation of argininosuccinate synthase (encoded by *argG*) and repressor ArgR combined with the overexpression of *argJ* encoding ornithine acetyltransferase in *C. glutamicum* ATCC13032 resulted in the L-citrulline production of 8.51 g/L [16]. The deletion of *argR* and *argG* genes combined with the overexpression of $argB^{fbr}$ (encoding feedback resistant NAGK) and *argF* (encoding ornithine carbamoylphosphate transferase) in *C. glutamicum* MB001 resulted in the accumulation of 7.7 g/L of L-citrulline in minimal medium [17].

In this study, we performed the optimization of L-citrulline biosynthesis operon in *C. glutamicum* for L-citrulline production. Firstly, we inserted the $argB_{Ec}$ gene at an appropriate site in gene cluster *argCJBDFRGH* to achieve the expression of feedback-resistant $NAGK_{Ec}$ and the deletion of *argR* and *argG* genes. Then, the plasmid-based overexpression of the optimized L-citrulline operon $argCJBDFB_{Ec}$ was carried out to further improve L-citrulline biosynthesis.

2. Materials and Methods

2.1. Microorganism and Cultivation Conditions

All strains plasmids [18,19] used in this study are listed in Table 1. *E. coli* strain JM109 was used as the host for plasmid construction and was cultured in Luria–Bertani (LB) medium at 37 °C. The precultivations of *C. glutamicum* strains were carried out in LBG medium (LB medium supplemented with 5 g/L glucose) at 30 °C. For L-arginine auxotroph strain precultivation, 0.5 g/L L-arginine was added to the LBG medium. Where appropriate, kanamycin (25 mg/L for *C. glutamicum* strains, 50 mg/L for *E. coli* strains) was added to the cultures.

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or Source
Strains		
E. coli JM109	General cloning host	TaKaRa
C. glutamicum ATCC13032	Wild-type strain	ATCC
CRB	*C. glutamicum* ATCC13032 derivative with insertion of $argB_{Ec}$ into *argR* gene	This work
CRBG	*C. glutamicum* ATCC13032 derivative with insertion of $argB_{Ec}$ between *argR* and *argG* genes	This work
CRBGC	CRBG derivative harboring pDXW-10-*argCJBDF*	This work
CRBGCo	CRBG derivative harboring pDXW-10-$argCJBDFB_{Ec}$	
plasmids		
pK18*mobsacB*	Kan^r; vector for in-frame deletions	Lab stock [18]
pK18RB	Derived from pK18*mobsacB*, harboring *argR*F-$argB_{Ec}$-*argR*R fragment	This work
pK18RBG	Derived from pK18*mobsacB*, harboring *argR*F′-$argB_{Ec}$-*argG*R′ fragment	This work
pDXW-10	Kan^r; shuttle vector between *E. coli* and *C. glutamicum*	Lab stock [19]
pDXW-10-*argCJBDF*	Derived from pDXW-10, for constitutive expression of *argCJBDF* gene cluster	This work
pDXW-10-$argCJBDFB_{Ec}$	Derived from pDXW-10, for constitutive expression of the optimized L-citrulline operon $argCJBDFB_{Ec}$	This work

For L-citrulline fermentation in shake flasks, the *C. glutamicum* strains from agar slants were inoculated in 20 mL seed medium (LBG medium), and 0.5 g/L L-arginine was added for the L-arginine auxotroph strain. Seed cultivation was carried out at 30 °C for 18 h in 250-mL shake flasks with a rotational speed of 220 r/min. The seed culture (1 mL) was transferred into 250-mL shake flasks containing 20 mL fermentation medium. The fermentation medium contained glucose 80 g/L, yeast extract 8 g/L, $(NH_4)_2SO_4$ 40 g/L, KH_2PO_4 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $FeSO_4{\cdot}7H_2O$ 0.02 g/L, $MnSO_4$ 0.02 g/L, and $CaCO_3$ 20 g/L; 0.5 g/L L-arginine was added for the L-arginine auxotroph strain, and the fermentation was carried out at 30 °C with a rotational speed of 220 r/min.

For L-citrulline fed-batch fermentation in the bioreactor, the *C. glutamicum* strain from agar slants was inoculated in a 50 mL seed medium (LBG medium supplemented with 0.5 g/L L-arginine) and cultured at 30 °C for 18 h in 500-mL shake flasks with a rotational speed of 220 r/min. The seed culture (100 mL) was transferred into 5-L stirred fermenter (BIOTECH-5BG, Baoxing Co., Shanghai, China) containing 1.5 L fermentation medium. The fermentation medium contained glucose 60 g/L, yeast extract 8 g/L, $(NH_4)_2SO_4$ 20 g/L, KH_2PO_4 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $FeSO_4{\cdot}7H_2O$ 0.02 g/L, $MnSO_4$ 0.02 g/L, and L-arginine 0.5 g/L. The feed medium contained glucose 700 g/L, yeast extract 8 g/L, KH_2PO_4 1.5 g/L, and $MgSO_4{\cdot}7H_2O$ 0.5 g/L and was fed into the fermenter when the residual glucose concentration was below 10 g/L. The temperature was maintained at 30 °C, and the pH was maintained at 7.0 by addition of 30% NH_4OH solution. The air flow rate was maintained at 2 L/min, and the agitation speed was 600 r/min.

2.2. Analytical Methods

Cell growth was monitored by measuring the OD_{600} using a spectrophotometer (UNICO™-UV2000, UNICO (Shanghai) Instrument Co., Ltd., Shanghai, China). For monitoring the cell growth during L-citrulline fermentation in shake flasks, the culture fluid was diluted by 0.2 mol/L HCl to dissolve $CaCO_3$. The glucose was measured using a bioanalyzer (SBA-40C, Biology Institute of Shandong Academy of Sciences, Jinan, China). The amino acids were measured by high-pressure liquid chromatography [20].

2.3. Transcriptional Analysis

The total RNA of *C. glutamicum* was extracted at the exponential phase using the RNAiso Plus reagent (Takara, Dalian, China), and the synthesis of cDNA was performed using the PrimeScript RT reagent kit (Takara, Dalian, China). Real-time PCR (RT-PCR) was carried out as described previously [21]. The primers used for the RT-PCR analysis of genes in the *argCJBDF* cluster were referred to the published study [22], and the primers EcBF (5′-GCCGTAGGTTTGTTTCTCGG-3′) and EcBR (5′-CGTTCATCAGTTGCCCTTC-3′) were used for the RT-PCR analysis of the $argB_{Ec}$ gene. The 16S rRNA relative abundance was used as an internal standard to standardize the results.

2.4. Construction of Plasmids and Strains

All of the plasmids used in this study are listed in Table 1, and the primers are listed in Table 2. The insertion of $argB_{Ec}$ into the *C. glutamicum* genome was performed via two-step homologous recombination using vector pK18*mobsacB* [18]. The plasmid pK18RB was used for the insertion of $argB_{Ec}$ into the *argR* gene. To obtain the *argR*F-$argB_{Ec}$-*argR*R fragment, the upstream and downstream regions of the *argR* gene (*argR*F and *argR*R) were amplified using the primer combinations RB-RF1/RB-RR1 and RB-RF2/RB-RR2, respectively, from genomic DNA of *C. glutamicum* ATCC13032; the integral $argB_{Ec}$ gene was amplified using the primers RB-BF and RB-BR from the genomic DNA of *E. coli* BL21; then, the *argR*F fragment and the $argB_{Ec}$ gene were fused in an overlap-extension PCR using the primers RB-RF1 and RB-BR, and the *argR*F-$argB_{Ec}$ fragment was obtained; and finally, the *argR*F-$argB_{Ec}$ and *argR*R fragments were fused using the primers RB-RF1 and RB-RR2, and the *argR*F-$argB_{Ec}$-*argR*R fragment was obtained. This *argR*F-$argB_{Ec}$-*argR*R fragment was cloned into pK18mobsacB, resulting in plasmid pK18RB. This plasmid was electro-transformed

into *C. glutamicum* ATCC13032, and the modified strain CRB was screened as described previously [11].

Table 2. Primers used in this study.

Names	Sequences (5′→3′)	Restriction Sites
RB-RF1	CCGGAATTCTAACGCTAACAAGCTCTCATTTTGCAGATT	*Eco*RI
RB-RR1	TTACATTTAGCGGCGCAGCTTCTCCGACGGAC	
RB-RF2	TAGCTTAAGTTTTGTTGGCCGGAGGCGCAGTGATGAACTGCTGGTTTCTACAGATC	
RB-RR2	CGCGGATCCGCGCCCGCTGAGTAATTCACCTA	*Bam*HI
RB-BF	AGCTGCGCCGCTAAATGTAACGAAGACAACTTACTGAAAG	
RB-BR	GGCCAACAAAACTTAAGCTA	
RBG-RF	CCGGAATTCGCACGCCAAGCTCTCATTTTGCAGATTTTG	*Eco*RI
RBG-RR	GGTGCGCAGCATCGCGATGTTGCCGG	
RBG-GF	GGCGCAGCTTTCCGGCATTGAATTTCGCTACAAGCGCGGCGTTGACGCACG	
RBG-GR	CGCGGATCCCTAAATACTTGAAGTTCTAGTCTGG	*Bam*HI
RBG-BF	CCGGCAACATCGCGATGCTGCGCACCCCAATATTCGTTTCGGCTATGCGGAAAC	
RBG-BR	GAAATTCAATGCCGGAAAGCTGCGCC	
CF	AAGGAAAAAAGCGGCCGCAAATTCATGCTTTTACCCACTTGCAG	*Not*I
CR	GGAAGATCTGCAAAATGAGAGCTTGGCGTGCAGTGCG	*Bgl*II
CoR	AAGGAAAAAAGCGGCCGCGAAATTCAATGCCGGAAAGCTGCGCC	*Not*I

The plasmid pK18RBG was used for the insertion of $argB_{Ec}$ between the *argR* and *argG* genes. The primers RBG-RF and RBG-RR were used for the amplification of the upstream region of the *argR* gene (*argR*F′), the primers RBG-GF and RBG-GR were used for the amplification of downstream region of the *argG* gene (*argG*R′), and the primers RBG-BF and RBG-BR were used for amplification of the integral $argB_{Ec}$ gene. Overlap-extension PCR was used for obtaining the *argR*F′-$argB_{Ec}$-*argG*R′ fragment as described above. This *argR*F′-$argB_{Ec}$-*argG*R′ fragment was cloned into pK18*mobsacB*, resulting in plasmid pK18RBG. Additionally, plasmid pK18RBG was used for CRBG strain construction as described above.

Plasmid pDXW-10 [19] was used for the constitutive expression of the *argCJBDF* and $argCJBDFB_{Ec}$ gene cluster. The *argCJBDF* gene cluster was amplified using the primers CF and CR from the genomic DNA of *C. glutamicum* ATCC13032. The resulting DNA fragment was cloned into pDXW-10, resulting in plasmid pDXW-10-*argCJBDF*. This plasmid was used to transform the CRBG strain using electroporation and to obtain the CRBGC strain. The $argCJBDFB_{Ec}$ gene cluster was amplified using the primers CF and CoR from the genomic DNA of the CRBG strain. The resulting DNA fragment was cloned into pDXW-10, and the restriction enzyme *Nhe*I was used to validate the direction of gene ligation. The correct ligating plasmid was named pDXW-10-$argCJBDFB_{Ec}$. This plasmid was used to transform the CRBG strain using electroporation and to obtain the CRBGCo strain.

3. Results and Discussion

3.1. Effects of argR/argG Deletion and $argB_{Ec}$ Expression on L-Citrulline Biosynthesis of C. glutamicum

In the CRB strain, the heterologous $argB_{Ec}$ gene encoding feedback-resistant $NAGK_{Ec}$ from *E. coli* was inserted into the *argR* gene in the genome (Figure 1). Therefore, the *argR* gene encoding the repressor ArgR of the *argCJBDFR* and *argGH* operons was disrupted. The expression of the genes in the *argCJBDF* and *argGH* operons was strengthened in the CRB strain at the transcriptional level (Figure 2). This result indicated that the expression of the *argCJBDF* and *argGH* operons in the CRB strain was derepressed by the inactivation of the ArgR repressor. Meanwhile, the transcriptional analysis revealed that the $argB_{Ec}$ gene was expressed along with the operons in the CRB strain (Figure 2).

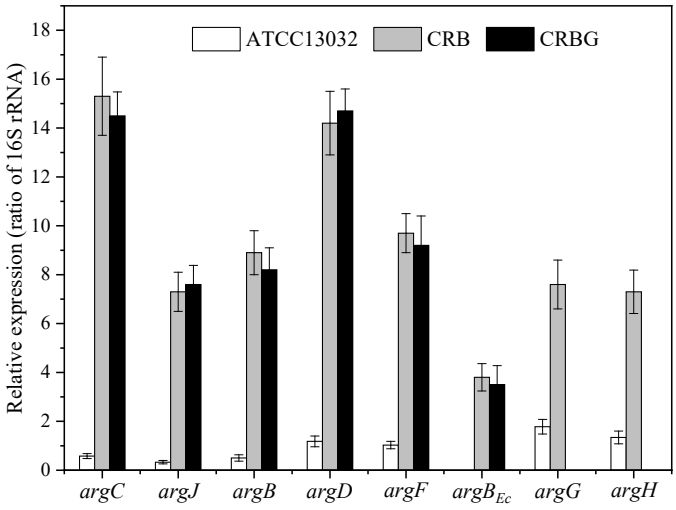

Figure 2. Transcriptional analysis of genes involved in L-citrulline and L-arginine biosynthesis. The expression levels of the genes were calculated as the ratio of 16S rRNA expression level. The error bars represent the standard deviation of three independent replicates.

As reported [13,17], *C. glutamicum* ATCC13032 could not accumulate L-citrulline and L-arginine (Figure 3a). Batch fermentation in the shake flasks revealed that the CRB strain could produce 3.6 g/L of L-citrulline and 1.1 g/L of L-arginine (Figure 3b). Previous research has shown that individually alleviating the feedback inhibition of NAGK by L-arginine or individually alleviating the transcriptional repression of the *argCJBDF* and *argGH* operons by the ArgR repressor could not make *C. glutamicum* ATCC13032 produce L-arginine [13]. Only simultaneously alleviating the feedback inhibition and transcriptional repression could give rise to excessive synthesis and the accumulation of L-arginine by *C. glutamicum* ATCC13032 [13]. The strain CRB could produce L-arginine; therefore, the insertion of $argB_{Ec}$ into the *argR* gene led to the expression of heterologous, feedback-resistant $NAGK_{Ec}$ and the inactivation of the ArgR repressor simultaneously in the CRB strain. In other words, the inactivation of the ArgR repressor and the expression of feedback-resistant $NAGK_{Ec}$ were achieved in one-step genome modification.

In the CRBG strain, the $argB_{Ec}$ gene was inserted into the genome between the *argR* and *argG* genes; meanwhile, the downstream region of the *argR* gene and the upstream region of the *argG* gene were deleted (Figure 1). Therefore, the ArgR repressor and argininosuccinate synthase were inactivated simultaneously. The expression of genes in the *argCJBDF* operon was strengthened in the CRBG strain at the transcriptional level (Figure 2). Meanwhile, the transcriptional analysis also revealed that the $argB_{Ec}$ gene was expressed along with the *argCJBDF* operon, in the CRBG strain (Figure 2).

Batch fermentation in shake flasks showed that the strain CRBG could produce 5.7 g/L of L-citrulline with a yield of 0.13 g/g glucose, and there was no accumulation of L-arginine in fermentation broth (Figure 3c). The results indicated that the conversion of L-citrulline to L-arginine was blocked in the CRBG strain by deleting the *argG* gene. Therefore, the inactivation of the ArgR repressor, the expression of feedback-resistant $NAGK_{Ec,}$ and the blocking of L-citrulline conversion were achieved simultaneously in the CRBG strain by one-step genome modification.

3.2. Further Enhancing of L-Citrulline Biosynthesis by Plasmid-Based Overexpression of the Optimized L-Citrulline Operon

In *C. glutamicum*, the *argCJBDF* operon encodes all of the enzymes for L-citrulline synthesis from L-glutamate. After the elimination of the transcriptional repression of *argCJBDF* operon by ArgR, the plasmid-based expression of L-citrulline operon can further increase the expression level. Thus, the plasmid-based overexpression of the L-citrulline operon strain CRBGC and CRBGCo was constructed. The batch fermentation of the CRBGC

(Figure 4a) and CRBGCo (Figure 4b) strain in shake flasks resulted in 8.2 g/L and 9.1 g/L of L-citrulline, respectively, with a yield of 0.16 g/g glucose and 0.18 g/g glucose, respectively, which were obviously higher than those of the CRBG strain (Figure 3c). In addition, the CRBGCo strain obtained higher L-citrulline production than the CRBGC strain. The results indicated that the L-citrulline biosynthesis in the CRBGC and CRBGCo strains was further strengthened by the plasmid-based overexpression of the L-citrulline operon, and the plasmid-based overexpression of the optimized L-citrulline operon was more beneficial to the L-citrulline biosynthesis.

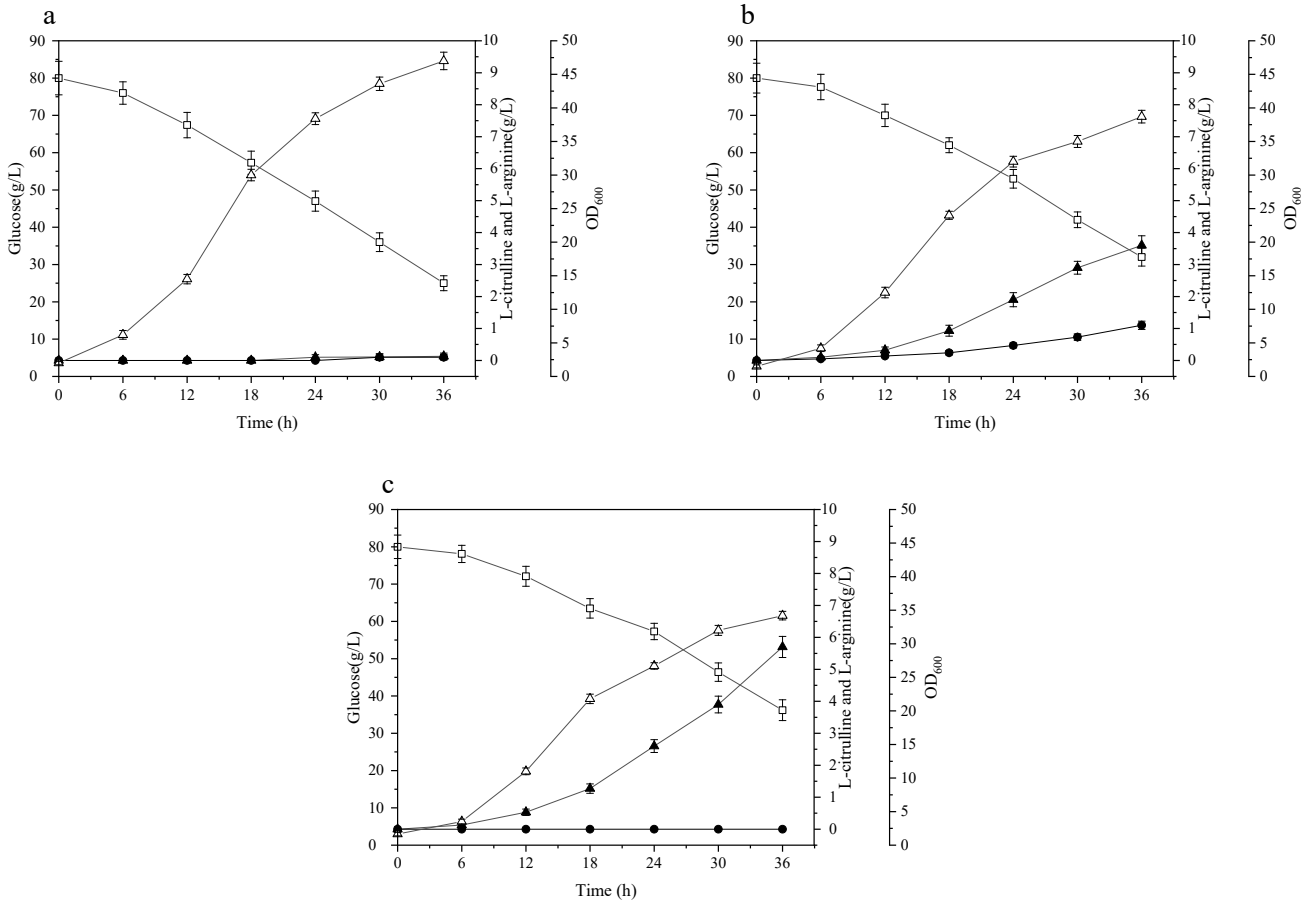

Figure 3. Batch fermentation of *C. glutamicum* ATCC13032 (**a**), CRB (**b**), and CRBG (**c**) strains in shake flasks. Signal denotes: filled triangle, L-citrulline; filled circle, L-arginine; empty square, glucose; and empty triangle, OD_{600}. The error bars represent the standard deviation of three independent replicates.

In L-ornithine-producing *C. glutamicum*, the plasmid-based overexpression of the *argCJBD* genes (Figure 1) could significantly increase the L-ornithine production [15]. Similarly, in L-arginine producing *C. crenatum*, the plasmid-based overexpression of the *argCJBDFRGH* cluster (Figure 1) could also strengthen the L-arginine synthesis [23]. In this study, the L-citrulline biosynthesis was also strengthened by the plasmid-based overexpression of the L-citrulline operon. Therefore, the intensification of the whole biosynthetic pathway is effective to increase the biosynthesis efficiency of the target product.

3.3. Fed-Batch Fermentation for L-Citrulline Production

The production performance of the CRBGCo strain was investigated in a fed-batch process, and the fermentation process is shown in Figure 5. The fed-batch fermentation of the CRBGCo strain resulted in 26.7 g/L of L-citrulline with a yield of 0.18 g/g glucose. The results indicate that the optimization of the L-citrulline operon in *C. glutamicum* is effective to construct the L-citrulline over-producing strain. In fed-batch fermentation, the L-citrulline yield from the glucose of the CRBGCo strain was the same as the batch

fermentation in shake flasks, but the productivity (0.42 g/L/h) was higher than that of the batch fermentation (0.25 g/L/h). In the next work, the production of L-citrulline by the CRBGCo strain can be improved through the optimization of the fermentation conditions.

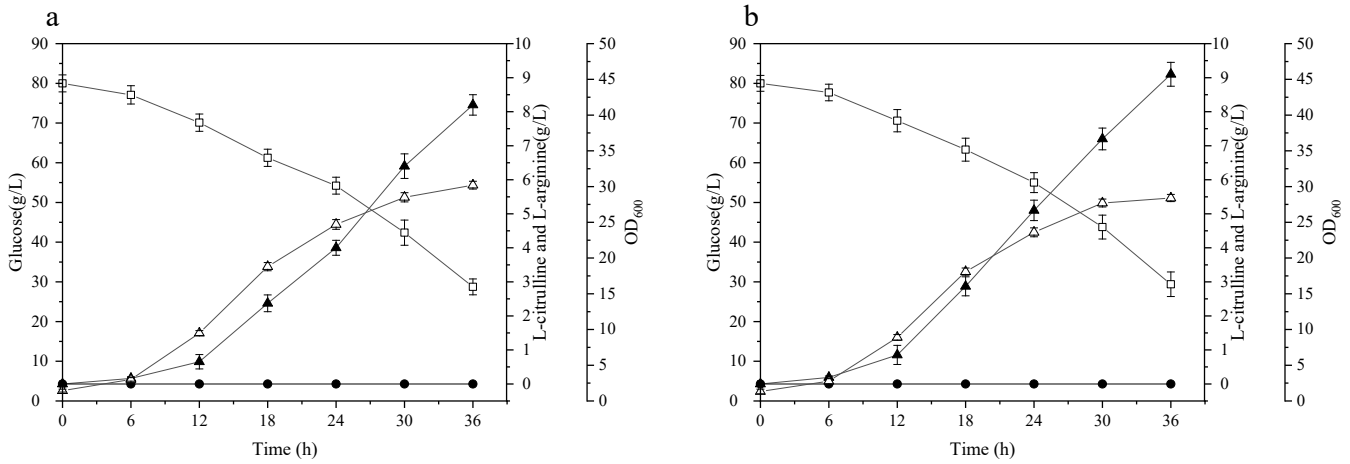

Figure 4. Batch fermentation of CRBGC (**a**) and CRBGCo (**b**) strains in shake flasks. Signal denotes: filled triangle, L-citrulline; filled circle, L-arginine; empty square, glucose; and empty triangle, OD_{600}. The error bars represent the standard deviation of three independent replicates.

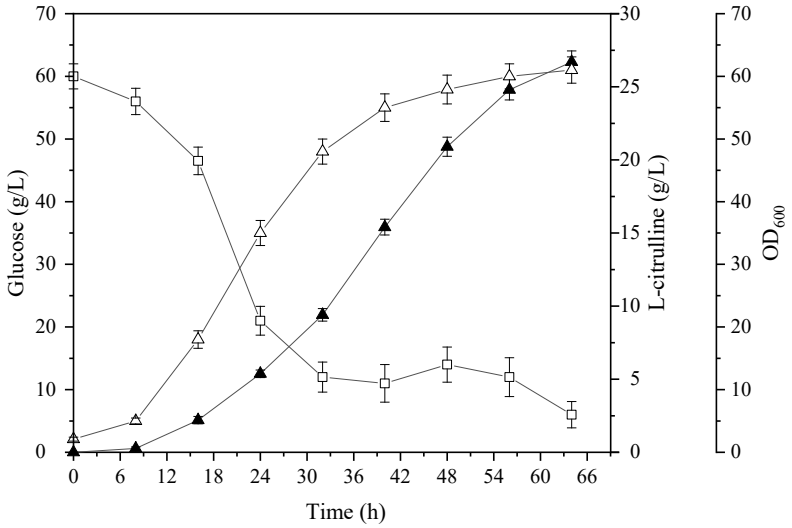

Figure 5. Fed-batch fermentation of CRBGCo strain in 5-L stirred fermenter. Signal denotes: filled triangle, L-citrulline; empty square, glucose; and empty triangle, OD_{600}. The error bars represent the standard deviation of three independent replicates.

4. Discussion

In this study, we performed preliminary metabolic engineering on *C. glutamicum* for L-citrulline production, and the work was focused on the optimization of the L-citrulline operon. The resulting CRBGCo strain could produce 26.7 g/L of L-citrulline in fed-batch fermentation. Therefore, as reported previously [16,17], the results in this study also prove that *C. glutamicum* can be used for L-citrulline fermentation production. Compared to the CIT0(pVWEx1-*argFB*^{fbr}) strain derived from *C. glutamicum* MB001 [17], the L-citrulline productivity and yield from the glucose of the CRBGCo strain in batch fermentation were relatively low, but the L-citrulline production of the CRBGCo strain was relatively high. Compared to the CIT strain derived from *C. glutamicum* ATCC13032 [16], the CRBGCo strain had higher L-citrulline production, productivity, and yield from glucose in the batch fermentation. Therefore, the metabolic engineering strategies used in this study are

effective for constructing L-citrulline-producing *C. glutamicum*. The feedback-insensitive NAGK is critical for L-citrulline synthesis in *C. glutamicum* [8,17]. Therefore, the L-citrulline synthesis pathway can be further fine-tuned by regulating the expression level of the $argB_{Ec}$ gene through the integration of different promoters [5,15]. *C. glutamicum* MB001, derived from *C. glutamicum* ATCC13032, a prophage-free *C. glutamicum* strain with a genome reduced by 6%, is more efficient for plasmid-based gene overexpression [17,24]. Therefore, the *C. glutamicum* MB001 is perhaps more suitable for the tuning of natural or synthetic metabolic pathways required for small-molecule production [24]. In the next work, the metabolic engineering strategies used in this study can be performed in *C. glutamicum* MB001, and more efficient L-citrulline production is possible to realize.

Similar to the previous study [17], in batch fermentation, the biomass of the L-citrulline-producing strains was decreased when L-citrulline production was increased (Figures 3 and 4). This is because more carbon flux and energy were used for L-citrulline production when the L-citrulline synthesis pathway was strengthened, and the cell growth was negatively affected. In batch fermentation, the initial concentration of glucose was set at a high level (80 g/L) to avoid the depletion of glucose, and the residual glucose concentration was around 30 g/L. Meanwhile, the productivity of L-citrulline in the fed-batch fermentation was obviously enhanced compared with that in the batch fermentation. Therefore, the L-citrulline production potential of the CRBGCo strain can be further unleashed through the optimization of the fermentation medium and conditions.

Similar to L-arginine, the biosynthesis of L-citrulline in *C. glutamicum* requires ATP, NADPH, and carbamoyl phosphate (Figure 1) [5,11,12,25]. Systems metabolic engineering, including the overexpression of biosynthetic genes, the removal of metabolism regulation [5], the increase in the supply of the L-glutamate precursor [12], cofactors [5,15], energy [11], the building block (carbamoyl phosphate) [5], and the improvement of the ammonia assimilation [25,26], can be carried out to optimize the metabolic network and obtain a more efficient L-citrulline-producing strain. *C. glutamicum* can use a variety of carbon sources for cell growth and product synthesis; more importantly, *C. glutamicum* can simultaneously utilize different carbon sources present in mixtures such as lignocellulosic hydrolysates [17]. In the next work, the investigation of using lignocellulosic hydrolysates as a carbon source for L-citrulline production can be performed.

5. Conclusions

In this study, the optimization of the L-citrulline operon in *C. glutamicum,* including the chromosomal integration of the heterologous $argB_{Ec}$ gene, the chromosomal deletion of the *argR* and *argG* genes, and the plasmid-based overexpression of the optimized L-citrulline operon, was performed for L-citrulline production. In *C. glutamicum* ATCC13032, the chromosomal integration of the integral $argB_{Ec}$ gene could introduce the natively insensitive NAGK, the deletion of the *argR* gene could relieve the repression of the L-citrulline operon by the ArgR repressor, and the deletion of the *argG* gene could block the conversion of L-citrulline to L-arginine. These three strain-engineering strategies were simultaneously achieved by one-step genome modification, and the L-citrulline-producing strain was obtained. Further enhancing the expression level of the optimized L-citrulline operon by plasmid-based overexpression could further improve the L-citrulline production. In the next work, systems metabolic engineering and fermentation-process optimization can be performed to increase the L-citrulline production, and using a non-food-derived carbon source such as lignocellulosic hydrolysates for L-citrulline production can also be investigated.

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