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Improvement of L-citrulline production in *Corynebacterium glutamicum* by ornithine acetyltransferase

N. Hao · Jr. Mu · N. Hu · S. Xu · M. Yan · Y. Li · K. Guo · L. Xu

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Abstract In this study, *Corynebacterium glutamicum* ATCC 13032 was engineered to produce L-citrulline through a metabolic engineering strategy. To prevent the flux away from L-citrulline and to increase the expression levels of genes involved in the citrulline biosynthesis pathway, the argininosuccinate synthase gene (*argG*) and the repressor gene (*argR*) were inactivated. The engineered *C. glutamicum* ATCC 13032 Δ*argG* Δ*argR* (CIT 2) produced higher amounts of L-citrulline (5.43 g/L) compared to the wildtype strain (0.15 g/L). To determine new strategies for further enhancement of L-citrulline production, the effect of L-citrulline on ornithine acetyltransferase (EC 2.3.1.35; OATase; ArgJ) was first investigated. Citrulline was determined to inhibit Ornithine acetyltransferase; for 50 % inhibition, citrulline concentration was 30 mM. The *argJ* gene from *C. glutamicum* ATCC 13032 was cloned, and the recombinant shuttle plasmid pXMJ19-*argJ* was constructed and expressed in *C. glutamicum* ATCC 13032 Δ*argG* Δ*argR* (CIT 2). Overexpression of the *argJ* gene exhibited increased OAT activity and resulted in a positive effect on citrulline production (8.51 g/L). These results indicate that OAT plays a vital role during L-citrulline production in *C. glutamicum*.

Keywords L-Citrulline · *Corynebacterium glutamicum* · Ornithine acetyltransferase · *argJ* · Feedback inhibition

N. Hao · Jr. Mu · N. Hu · S. Xu · M. Yan · Y. Li · K. Guo · L. Xu (✉)
State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, 30 Puzhu South Road, Nanjing 211816, People's Republic of China
e-mail: xulin@njtech.edu.cn

Introduction

Corynebacterium glutamicum is a gram-positive soil bacterium with high GC content [21]. As a workhorse of industrial microbiology, *C. glutamicum* has been widely used for the production of amino acids, primarily L-glutamate and L-lysine [5, 16]; however, it can also be used to produce arginine, ornithine, and citrulline.

L-citrulline is an intermediate metabolite in the arginine biosynthesis pathway, and as a non-standard α-amino acid, citrulline has a wide range of potential functions in different fields. Studies have reported that citrulline is a key intermediate in the urea cycle, which is used by mammals to excrete ammonia [6]. Citrulline is also widely used in health and nutrition applications [15]. Compared to the extraction of natural products [18] and enzymatic conversion [12], microbial fermentation [8] is the most promising method for L-citrulline production.

There are three routes for the biosynthesis of L-citrulline from the precursor L-glutamate in different organisms [3, 14, 24]. L-citrulline is biosynthesised through a six-step cyclic pathway (Fig. 1) that includes a series of acetylated intermediates in *C. glutamicum*. In this pathway, citrulline is converted to arginine by argininosuccinate synthase (*argG*) and arginosuccinase (*argH*) [3] and is regulated by the repressor ArgR shown to bind regions upstream of *argC*, *argB*, *argF*, and *argG* [19].

In the reaction, the acetyl group from acetylornithine in the citrulline biosynthetic pathway of *C. glutamicum* is recycled by ornithine acetyltransferase (OAT, ArgJ), which catalyses acetylornithine and glutamate into ornithine and *N*-acetylglutamate. ArgJ operates as a bifunctional protein and uses both substrates; this enzyme exhibits both NAGS (*argA*-encoded in *Escherichia Coli*) and OAT (*argE*-encoded in *E. coli*) activity. The earliest studies by

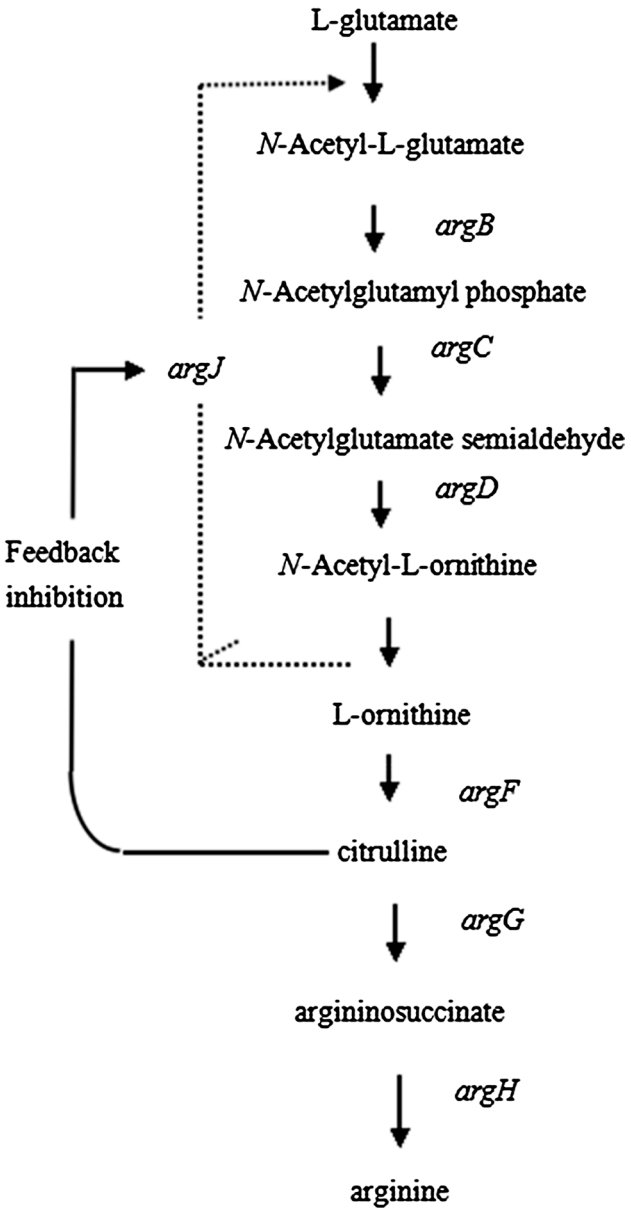

Fig. 1 Biosynthetic pathway of L-citrulline. *argB* acetylglutamate kinase, *argC* acetylglutamate semialdehyde dehydrogenase, *argD* acetylornithine transaminase, *argJ* ornithine acetyltransferase, arg*F* ornithine transcarbamylase, *argG* argininosuccinate synthase, *argH* arginosuccinase

Udaka and Kinoshita recognised that acetylated compounds form a cycle of reactions [23], whereas Sakanyan et al. [19] discovered that *C. glutamicum* only possesses a monofunctional ArgJ, which exhibits OAT activity but lacks NAGS activity. Recently, overexpression of the *argJ* gene showed increased NAGS activity by complementing the *C. glutamicum* arginine auxotrophic *argJ* strain [7]. Petri et al. [17] discovered that the monofunctional ArgJ only catalyses the fifth step of the citrulline biosynthesis pathway in *C. glutamicum,* and glutamate was acetylated by *N*-acetylglutamate synthase Cg3035. Enzyme inhibition tests showed that L-arginine had no influence on OAT activity; however, the ArgJ enzyme was inhibited when 5 mM L-ornithine was added [19].

In this study, *C. glutamicum* ATCC 13032 was engineered to produce citrulline by deleting the *argG* gene [21]; furthermore, the feedback repression by the arginine repressor (ArgR) was relieved. To investigate the inhibition of L-citrulline on OATase activity, the *argJ* gene from *C. glutamicum* ATCC 13032 was cloned and expressed to study its effects on OAT activity and L-citrulline production. Our results demonstrated that L-citrulline overproduction in the engineered strain correlated with expression levels of ArgJ, which plays a vital role in the L-citrulline biosynthesis in *C. glutamicum.*

Materials and methods

Strains, plasmids and primers

The strains, plasmids, and primers used in this study are listed in Tables 1 and 2.

Plasmid and strain construction

Gene *argJ* was amplified by PCR from the *C. glutamicum* ATCC 13032. After digestion with *Bam*HI and *Eco*RI, the amplified product was ligated into the *C. glutamicum*/*E. coli* shuttle vector pXMJ19 to create pXMJ19-ec *argJ*. The recombinant plasmid was transformed into *E. coli* DH5α and *C. glutamicum*. To construct the *C. glutamicum* mutant strain with the *argR* gene deletion, crossover PCR was used to generate the *argR* deletion fragment. This strategy involved the replacement of a segment of the *argR* gene from *C. glutamicum* with a short synthetic fragment that maintained the translational reading frame. The 5′-upstream region of *argR* gene was amplified by PCR from *C. glutamicum* ATCC 13032 chromosomal DNA using primers *argR*-up-F and *argR*-up-R, as well as the 3′-upstream region of *argR* gene was amplified using primers *argR*-down-F and *argR*-down-R. Crossover PCR was carried out to generate the *argR* deletion fragment using primers *argR*-up-F and *argR*-down-R. The resulting *argR* deletion fragment was digested with *Eco*RI and *Hin*dIII and subsequently ligated to the suicide vector pK18 *mobsacB*. The recombinant plasmid pK18 *mobsacB* Δ*argR* was transformed into *C. glutamicum* through electroporation. Double homologous recombination was performed as described by Schafer et al. [20]. PCR and DNA sequencing verified deletion of the target genes in pK18 *mobsacB* derivatives and the *C. glutamicum* recombinants.

Table 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristics	Source/references
Escherichia coli		
E. coli DH5α	*upE44 hsdR17 recA1 endA1 gyrA96 thi1 relA mcrA* Δ (*mrr-hsdRMS-mcrBC*)	Novagen
Corynebacterium glutamicum		
C. glutamicum ATCC 13032	Wild type, auxotrophic for biotin	American type culture collection
CIT 1	Wild-type *C. glutamicum* ATCC 13032 with deletion of *argG*, encoding argininosuccinate synthase, auxotrophic for L-arginine and biotin	Stored in lab
CIT 2	CIT 1 with deletion of *argR*, auxotrophic for L-arginine and biotin	This study
CIT 3	CIT 2 with expression *argJ* from *C. glutamicum* ATCC 13032, auxotrophic for L-arginine and biotin	This study
Plasmid		
pXMJ19	KanR, *E. coli–C. glutamicum* shuttle vector P_{lac}, $lacI^q$, Cm^R	Jakoby et al. [9]
pXMJ19-ec *argJ*	Carrying *argJ* from *C. glutamicum* ATCC 13032	This study
pK18 *mobsacB*	Plasmid for deletion mutagenesis, *oriV*, *oriT*, *mob*, *sacB*, *Kmr*	Schäfer et al. [20]
pK18 *mobsacB* Δ*argG*	pK18 *mobsacB* with 900-bp deletion of *argG*	Stored in lab
pK18 *mobsacB* Δ*argR*	pK18 *mobsacB* with 300-bp deletion of *argR*	This study

Table 2 Primers used in this study

Primer name	Sequence (5′ → 3′)[a]	Restriction enzyme
argG-up-F	GAAGAATTCGACACCGTTTTCGTTCTCG	*Eco*RI
argG-up-R	CCTGCGTGCAGGCACGCTGGCGAACGTTGTCCATG	
argG-down-F	GCCAGCGTGCCTGCACGCAGGTTCCATCACCATC	
argG-down-R	GTTAAGCTTGGGTTGCCACCTGGTCG	*Hin*dIII
argR-up-F	GAAGAATTCGAGCGCGCGGAAAAGC	*Eco*RI
argR-up-R	CGGTGTCATCACAGCAGCAATTCAGACAGTTGTACC	
argR-down-F	GAATTGCTGCTGTGATGACACCGTTTTCGTTCTCG	
argR-down-R	GTTAAGCTTCTCTCCACCCTGGCCCA	*Hin*dIII
argJ-cg-F	CGCGGATCCCCCTAACCAGCAAACACAAC	*Bam*HI
argJ-cg-R	CCGGAATTCTCAGCGAGGACATTTGCG	*Eco*RI

[a] Restriction endonuclease sites existing in primer sequences are underlined

Bacterial strains and growth conditions

E. coli DH5α and *C. glutamicum* ATCC 13032 were used to construct the plasmid and the mutant strains, respectively, used in this study. *E. coli* and *C. glutamicum* were grown aerobically at 37 and 30 °C, respectively, in Luria–Bertani (LB) medium or LB medium with 10 % sucrose [20]. For L-citrulline production of *C. glutamicum*, the seed medium consisted of (per litre) 20 g glucose, 1.5 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g KH_2PO_4, 0.4 g $MgSO_4{\cdot}7H_2O$, 2.5 g urea, 0.02 g $MnSO_4{\cdot}H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, 100 μg biotin, 200 μg vitamin B1, and 100 mg arginine. The culture medium consisted of (per litre) 80 g glucose, 1.0 g $K_2HPO_4{\cdot}3H_2O$, 1.0 g KH_2PO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 40 g $(NH_4)_2SO_4$, 0.02 g $MnSO_4{\cdot}H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, 1 mg $ZnCl_2$, 0.2 mg $CuSO_4$, 100 μg biotin, 200 μg vitamin B1, 30 g $CaCO_3$, and 100 mg arginine. The initial pH of all the above media was adjusted to 7.0. Shake flask cultures were prepared to test the effects of *argJ* on L-citrulline production. A 5-ml sample of the seed culture previously grown for 12 h was inoculated into 30 ml of the culture medium in a 500-ml flask and cultured for 72 h. All cultures were grown at 30 °C and shaken at 200 rpm on a rotary shaker. When necessary, 50 mg/L kanamycin or 20 mg/L chloramphenicol was added in the *E. coli* medium; 25 mg/L kanamycin or 10 mg/L chloramphenicol was added in the *C. glutamicum* medium.

Activity assay

Ornithine acetyltransferase (ArgJ), *N*-acetylglutamate kinase (ArgB), *N*-acetylglutamate semialdehyde dehydrogenase (ArgC), acetylornithine transaminase (ArgD), and ornithine transcarbamylase (ArgF) activities were detected according to the methods of Liu et al. [13], Udaka [22], Chun et al. [2], Friedrich et al. [4], and Kumar et al. [11], respectively.

C. glutamicum cells were grown in LB medium, harvested by centrifugation during the exponential phase, and washed in 100 mM Tris/HCl buffer (pH 7.5). Crude cell extracts were prepared by sonic disruption. All treatments were performed at 4 °C. Protein concentration was determined by the Bradford method [1]. OAT activity was measured in cell extracts by spectrophotometric determination of the formation of ornithine at 470 nm [13]. Assays were performed in a total volume of 0.5 ml containing 100 mM Tris–HCl buffer (pH 7.5), 60 mM *N*-Acetylornithine, 60 mM Glutamate and 200 µl cell extracts at 37 °C for 10 min. Next, the reaction was terminated by adding 500 µl volume of ninhydrin reagent (0.4 M citric acid 1 % ninhydrin in a methoxyethanol, 1:2, by vol.) and heated at 100 °C for 10 min. 500 µl volume of NaOH (4.2 M) were added and incubated for 20 min at room temperature. Subsequently, the concentration of ornithine produced was measured at 470 nm and determined by reference to a standard curve of 0–2,000 nmol omithine. One unit of OAT was defined as the amount of enzyme that catalyses the formation of 1 µmol of ornithine per min under assay conditions.

Assays of cell concentration, glucose and L-citrulline

After diluting the culture with 0.2 mol/L HCl to dissolve $CaCO_3$, cell growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}) using a spectrophotometer and converted to the cell dry weight (CDW), an OD_{600} of 1 was determined to equal 0.25 g CDW/L [10]. The glucose concentration was determined using a biosensor (Institute of Biology, Shandong Academy of Science, Shanghai, China). The Venusil-AA analytical method (Agela Technologies, Beijing) was used to determine the concentration of L-citrulline with pre-column derivatisation by phenyl isothiocyanate. A Dionex UltiMate 3000 series HPLC system was used for this study. Data collection and integration were performed using the Chromeleon Client software (version 6.80, build 2212).

Recombinant strains stability assay

To test for stability of the recombinant strains (CIT 1, CIT 2, CIT 3), the culture procedure was repeated continuously for approximately 10 generations, and citrulline concentrations were determined.

Results

Metabolic engineering of *C. glutamicum* for L-citrulline production

To construct a host strain capable of accumulating L-citrulline, it was first focused on inhibiting citrulline degradation of L-citrulline into arginine in *C. glutamicum* ATCC 13032 [21]. Thus, the *argG* gene encoding argininosuccinate synthase was deleted. The resulting strain of *C. glutamicum* ATCC 13032 Δ*argG* was named CIT 1. Functional verification indicated that CIT 1 is an arginine-requiring auxotrophic mutant, which used arginine for its growth. CIT 1 was cultivated in the medium and produced 2.52 g/L L-citrulline (Table 3). Compared to the wild-type *C. glutamicum* ATCC 13032 (0.15 g/L), the CIT 1 could accumulate a certain amount of L-citrulline.

Table 3 Accumulation of L-citrulline in *C. glutamicum* ATCC 13032 derivatives

strain	Citrulline[a] (g/L)
C. glutamicum ATCC 13032	0.15 ± 0.04
CIT 1	2.52 ± 0.10
CIT 2	5.43 ± 0.16
CIT 3	8.51 ± 0.61

[a] The accumulation of amino acids presented is described in mean ± standard deviation for triplicate experiments and statistically analysed at a level of $p < 0.05$

Feedback repression is a type of metabolic regulation that usually affects amino acid metabolism. Several reports on the mechanism of ArgR repressors indicate that the expression levels of the related genes in the arginine operon are affected by the binding of these repressors to the corresponding promoter regions in *C. glutamicum* [25]. For the citrulline biosynthesis of *C. glutamicum*, an auxotrophic mutant with an additional deletion of the transcriptional regulator of citrulline biosynthesis was constructed. The *argR* gene was deleted to amplify citrulline biosynthetic flux. The resulting strain of *C. glutamicum* Δ*argG* Δ*argR* was named CIT 2. The specific activities of the citrulline biosynthesis enzymes (ArgC, ArgJ, ArgB, ArgD, and ArgF) were significantly improved by measuring the crude cell-free extracts of the recombinant strain CIT 2 (Table 4). This finding indicated that CIT 2 had higher expression levels of the citrulline biosynthesis genes when compared to CIT 1. Meanwhile, it is found that the double knockout strain produces L-citrulline concentration up to 5.43 g/L (Table 3), which is higher than that (2.52 g/L) in CIT 1. The deletion of the *argG* and *argR* genes enhanced L-citrulline production in *C. glutamicum.* Thus, the ArgR protein functions during the feedback repression of citrulline synthesis in *C. glutamicum*.

Effects of L-citrulline on OAT activity

The *C. glutamicum* gene (*argJ*) encoding the enzyme, which deacetylates acetylornithine in the citrulline biosynthetic pathway, was cloned. OAT activity was inhibited by

Table 4 Specific activities of the enzymes in CIT 1 and CIT 2

Strains	Genotype	Specific activity (U/mg protein)				
		ArgJ	ArgB	ArgC	ArgD	ArgF
CIT 1	Δ*argG*	0.002 ± 0.0001	0.26 ± 0.02	2.56 ± 0.01	0.012 ± 0.001	0.35 ± 0.01
CIT 2	Δ*argG*, Δ*argR*	0.004 ± 0.0002	1.57 ± 0.08	4.24 ± 0.11	0.022 ± 0.005	1.12 ± 0.08

The data represent the mean of three independent cultures ± standard deviation

ArgJ ornithine acetyltransferase, *ArgB* acetylglutamate kinase, *ArgC* acetylglutamate semialdehyde dehydrogenase, *ArgD* acetylornithine transaminase, *ArgF* ornithine transcarbamylase

low concentration of citrulline, and arginine had no inhibition on the activity of OAT. Figure 2 presents the effect of citrulline on OAT activity. The assays using a crude extract of CIT 2 showed that the citrulline concentration for 50 % inhibition of OAT was 30 mM.

The specific activity of OAT from *C. glutamicum* was determined using crude extracts of the recombinant CIT 2. The OAT enzyme from *C. glutamicum* was sensitive to the end product L-citrulline. From these results, the *argJ* gene in *C. glutamicum* was targeted for the production of L-citrulline.

Effect of overexpression of *argJ* gene on L-citrulline production

OAT was subjected to feedback inhibition by L-citrulline in *C. glutamicum*. OAT overexpression may overcome this inhibition, thereby increasing the production of citrulline in CIT 2. To investigate the effect of overexpression of OAT on L-citrulline production in *C. glutamicum*, the pXMJ19-*argJ* plasmid carrying the *argJ* gene from *C. glutamicum* ATCC 13032 was constructed and transformed into *C. glutamicum* CIT 2. The resulting strain was named CIT 3. The overexpression of the *argJ* gene produced a large amount of L-citrulline (8.51 g/L) (Table 3). Citrulline production in the flask cultures was significantly increased, as expected. The enhanced production of citrulline under *argJ* homologous overexpression suggests that it was likely due to an increase in the expression level of OAT, which is inhibited by L-citrulline. The strain CIT3 (*C. glutamicum* ATCC 13032 Δ*argG* Δ*argR* pXMJ19-cg *argJ*) could be used as a basis to further improve L-citrulline production.

Stability of recombinant strains

Citrulline yields were measured to test the stability of the recombinant strains, which were cultured continuously for 10 generations in shake flasks. The citrulline concentrations of CIT 1, CIT 2 and CIT 3 are maintained at 2.50, 5.40 and 8.50 g/L, respectively, indicating that the recombinant strains have good genetic stability.

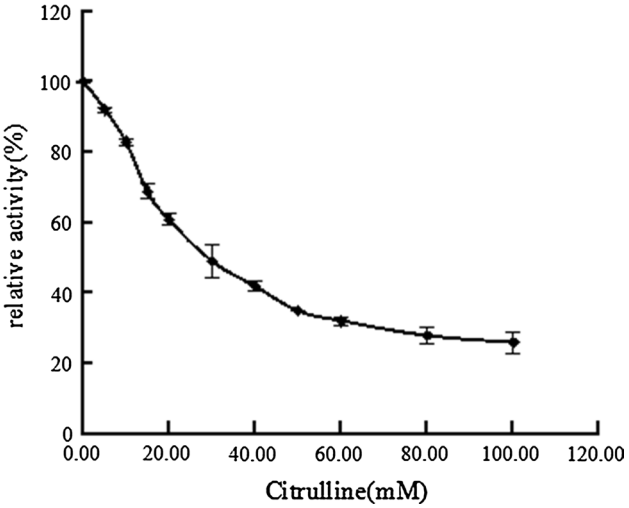

Fig. 2 Inhibition of OATase (*argJ*) activity of the *C. glutamicum* ATCC 13032 by L-citrulline. The data represent the mean of three independent cultures ± standard deviation

Discussion

Based on the wild-type *C. glutamicum* ATCC 13032, the strains CIT 1 (*C. glutamicum* ATCC 13032 Δ*argG*), CIT 2 (*C. glutamicum* ATCC 13032 Δ*argG* Δ*argR*) and CIT 3 (*C. glutamicum* ATCC 13032 Δ*argG* Δ*argR* pXMJ19-cg *argJ*) were obtained.

There was a 56.7 % increase in citrulline production from CIT 2 (5.43 g/L) to CIT3 (8.51 g/L). CIT 3 produced threefold higher citrulline than CIT 1 (2.52 g/L). Overexpression of *argJ* gene indeed enhanced the metabolic flux of the L-citrulline biosynthetic pathway.

There were no differences in the cell growth and glucose consumption between *C. glutamicum* CIT 1, CIT 2 and CIT 3 (Fig. 3). OAT activity was measured in crude extracts of the constructed strains (Table 5). The highest OAT activity exceeded the activity in *C. glutamicum* CIT 1 8.5 times. As expected, overexpression of the *argJ* gene enhanced the weak expression, improved the activity, and reduced the inhibition of citrulline, and a significant increase in production could be achieved.

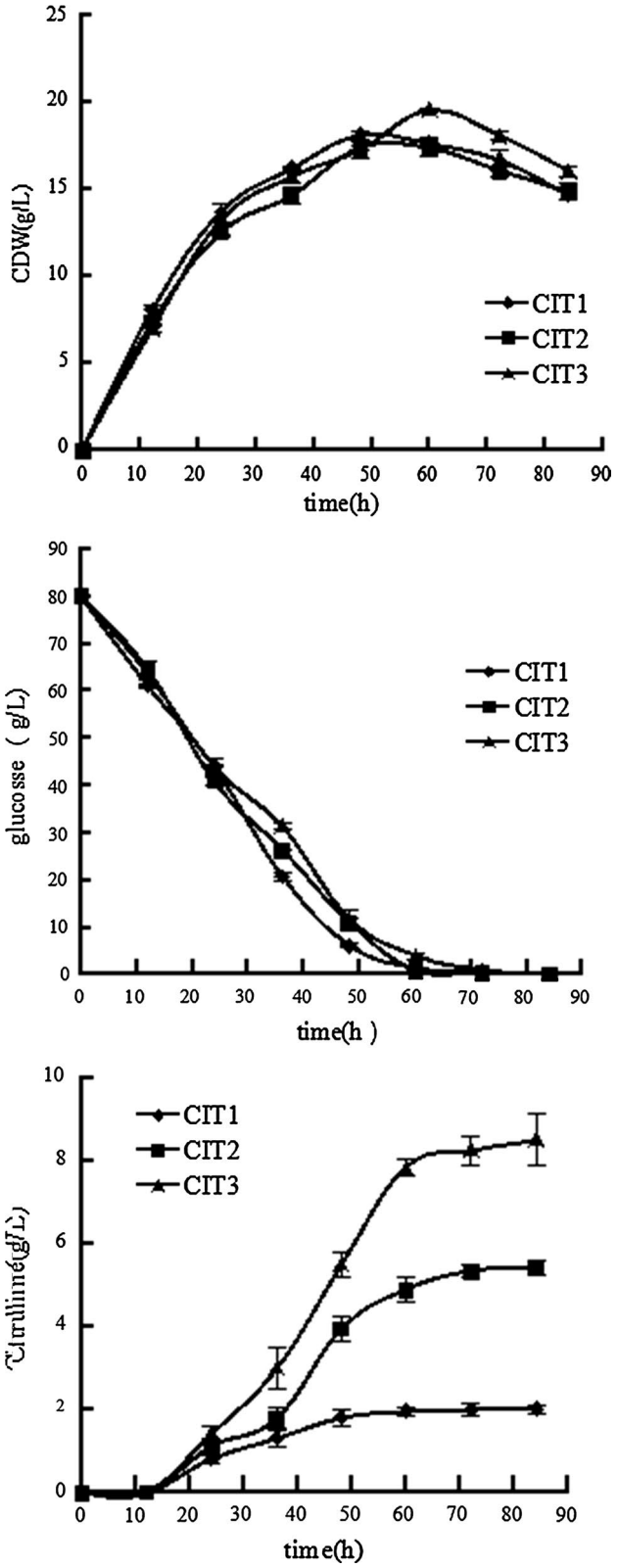

Fig. 3 Cell dry weight (CDW) and glucose concentration and citrulline production of *C. glutamicum* ATCC 13032 derivatives in culture. The data represent the mean of three independent cultures ± standard deviation

Table 5 Specific activities of the ornithine acetyltransferase in *C. glutamicum* ATCC 13032 derivatives

Crude extract	Specific activity (units/mg protein)[a]
C. glutamicum ATCC 13032	–
CIT 1	0.002
CIT 2	0.004
CIT 3	0.017

In summary, these results have clearly demonstrated that OAT does influence the production of citrulline in *C. glutamicum*. OAT is a key enzyme for enhancing the biosynthesis of citrulline from glutamate in *C. glutamicum*. Overexpression of the *argJ* gene from *C. glutamicum* was beneficial for achieving higher levels of citrulline production. Based on our measurements, in future studies, the *argJ* gene will be studied continuously to determine the mechanism of feedback inhibition.

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