Biotechnology and Bioprocess Engineering 17: 16-21 (2012)
DOI 10.1007/s12257-011-0360-8

RESEARCH PAPER

Construction of Recombinant *Corynebacterium glutamicum* for L-threonine Production

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Received: 7 August 2011 / Revised: 4 October 2011 / Accepted: 4 October 2011

Abstract L-threonine is an essential amino acid which is widely used in feed and pharmaceutical industries. We recently engineered *Corynebacterium glutamicum* R102 (AHV^r) for improved production of L-threonine. Inactivation of genes *metX* and *dapA* encoding dihydrodipicolinate synthase and homoserine O-acetyltransferase, respectively, was firstly conducted by homologous recombination, which differed from the common random mutagenesis method. Then operon gene *hom-thrB* (O) and export *gene thrE* (E) from R102 were over-expressed alone or together to obtain a series of recombinant strains. qPCR was employed to evaluate the transcript quantification of the target genes. In flask fermentation, the newly constructed strain R102Δ *metXΔdapA* (pEC-Box) was able to accumulate 3.35 g threonine/L compared with 1.80 g threonine/L of strain R102 (AHV^r).

Keywords: *Corynebacterium glutamicum*, L-threonine operon, threonine export, L-threonine production, *dapA*

1. Introduction

Due to their advantages of robust growth and clear genetic background, *E. coli* strains are the main producers of L-threonine at present. However, because of their endotoxins, *E. coli* strains are not appropriate for pharmaceutical-grade amino acid production [1]. *Corynebacterium glutamicum*, as the main strain for the production of L-glutamate and L-lysine so far [2,3], also has the potential to produce L-threonine. However, it has a different biosynthetic pattern than that of the most common producer *E. coli*.

As shown in Fig. 1 [4], L-threonine is synthesized from aspartate in a pathway comprising five reactions, catalyzed by aspartate kinase (EC2.7.2.4, *lysC* gene product), ASA dehydrogenase (EC1.2.1.11, *Asd* gene product), homoserine dehydrogenase (EC1.1.1.3, *Hom* gene product), homoserine

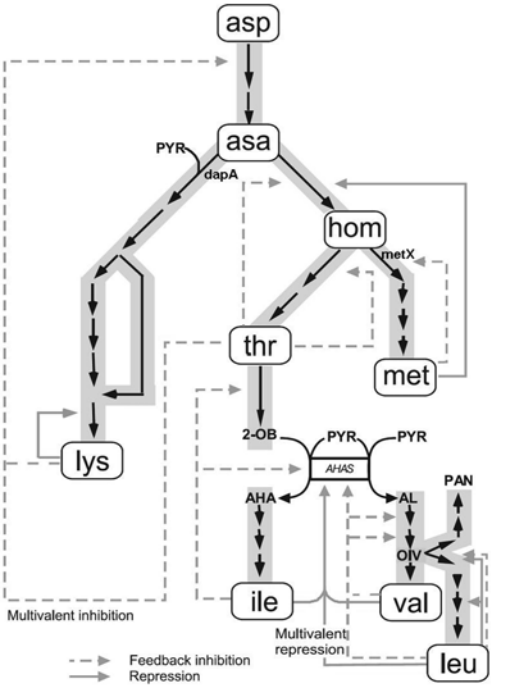

Fig. 1. Biosynthesis and regulation of the aspartate-derived amino acids in*Corynebacterium glutamicum*. Asp, aspartic acid; asa, aspartate semialdehyde; hom, homoserine; thr, threonine; met, methionine; lys, lysine; ile, isoleucine; val, valine; leu, leucine; 2-OB, 2-oxobutanoate; AHA, acetohydroxy acid; PYR, pyruvate; AL, acetolactate; OIV, oxoisovalerate; PAN, pantothenate; HDH, homoserine dehydrogenase; HK, homoserine kinase; AHAS, acetohydroxyacid synthase.

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kinase (EC2.7.1.39, *thrB* gene product), and threonine synthase (*thrC* gene product). At the branch point of aspartate semialdehyde, the ASA can be consumed by dihydrodipicolinate synthase (*dapA* gene product) to synthesize lysine. Also, homoserine O-acetyltransferase (*metX* gene product) competes for homoserine to synthesize methionine.

In *Corynebacterium glutamicum* (WT), aspartokinase encoded by *lysC* is feedback inhibited by lysine plus threonine; homoserine dehydrogenase is repressed by methionine. While in *E. coli*, three aspartokinase isoenzymes AKI, AKII, AKIII (encoded by *thrA*, *metL* and *lysC*) are inhibited by Thr, Met and Lys. (seen in KEGG database) Aspartate kinase I and II are also named homoserine dehydrogenase I and II, because they are bifunctional enzymes with two catalytic domains, one for aspartate kinase activity and the other for homoserine dehydrogenase activity. Threonine operon in *C. glutamicum* is constituted by two genes (*hom* and *thrB*), and this differs from that of *Escherichia coli* comprising three genes (*thrA*, *thrB*, *thrC*). The export gene encoded by *thrE* in *C. glutamicum* also differs from the exporters of *E. coli* encoded by *rhtA*, *rhtB*, and *rhtC* [5].

In our research, *metX* and *dapA* were knocked out in *C. glutamicum* R102 (AHV^r) [6] through homologous recombination instead of the common random mutation method. R102Δ*metX*, R102Δ*dapA*, and R102Δ*metX*Δ*dapA* were constructed subsequently. The operon genes *hom-thrB* (O) and export gene *thrE* (E), cloned from *C. glutamicum* R102 (AHV^r), were then overexpressed alone or together to obtain a series of recombinant strains.

Relative expression of the key genes and threonine yield in recombinant strains were investigated to evaluate the systematic strategy for threonine production in *C. glutamicum*.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and oligonucleotides

All bacterial strains and plasmids and their relevant characteristics and sources are listed in Supplementary Table 1. The main oligonucleotides used and their sequences are listed in Supplementary Table 2.

2.2. Construction of plasmids and strains

The isolation of plasmids from *E. coli* was performed by a plasmid extraction kit (Takara). The isolation of chromosomal DNA from *C. glutamicum* was performed as described previously [7]. Plasmid DNA transfer into *C. glutamicum* was carried out by electroporation [8]. *E. coli* was transformed with the $CaCl_2$ method.

Inactivation of the *metX*, *dapA* genes in R102 was performed using crossover PCR and the suicide vector pK18mobsacB [9]. The recombinant strains were also indentified by PCR. The over-expression recombinant strains are listed as follows:

a: two genes under one promoter (Figs. 2A and 2B), including R102Δ*metX*Δ*dapA* (pEC-O-E), R102Δ*metX*Δ*dapA* (pEC-E-O);

b: each gene under lac promoter (Fig. 2C) R102ΔΔ (pEC-Box) in short of R102Δ*metX*Δ*dapA* (pEC-p-O-p-E); the promoter of the plasmid itself was not removed.

2.3. Media and growth conditions

Seed and fermentation medium for *C. glutamicum* were adjusted according to the previous patent [10]. The initial OD600 was controlled at 0.1 in seed cultivation. After 22 h cultivation (30°C, 250 rpm.), the seed was transferred

Table 1. Strains and plasmids

Strain and plasmids	Relevant characteristics	Source or ref.
E.coli TOP10	F-, mcrAΔ (mrr-hsd RMS-mcrBC), ϕ80, lacZΔM15, ΔlacX74, recA1, araΔ139Δ (ara-leu)7697, galU, galK, rps, (Strr) endA1, nupG	Our lab
R102(AHV^r)	Derived from ATCC13032	A. J. Sinskey, 1991
R102ΔmetX	R102 with deletion in the *metX* gene	This work
R102Δ*dapA*	R102 with deletion in the *dapA* genes	This work
R102Δ*metX*Δ*dapA*	R102 with deletion in both *metX* , *dapA* genes	This work
pk18mobsacB	Km^r, mobilizable (*oriT*), *oriV*	Andreas Schgfer, 1994
pEC-T18mob2	*E. coli-C. glutamicum* shuttle vectors(Tet^r)	Andreas Tauch
pEC-hom-thrB(pEC-O)	Plasmid carrying Threonine operon (*hom-thrB*)	This work
pEC-thrE(pEC-E)	Plasmid carrying Threonine export gene (*thrE*)	This work
pEC-hom-thrB-thrE	Plasmid carrying Threonine operon (*hom-thrB*) and export gene (*thrE*)	This work
pEC-thrE-hom-thrB	Plasmid carrying export gene (*thrE*) and Threonine operon (*hom-thrB*)	This work
pEC-p-hom-thrB-p-thrE	Plasmid carrying Threonine operon (*hom-thrB*) and export gene (*thrE*). Each gene under lac promoter, promoter of the plasmid was not removed.	This work

Corynebacterium glutamicum was cultivated aerobically on a rotary shaker (250 rpm) at 30°C in Luria-Bertani (LB) medium with 2% (w/v) glucose. *E. coli* top10 was cultivated at 37°C in LB or plated onto LB agar plates.

Table 2. Main oligonucleotides used in this study

Main oligonucleotides	sequences	purpose
O-Lac promoter-1	ATAGAATTCACGCCAGCAACGC	Primers for plasmid pEC-Box construction
O-Lac promoter-2	GTAATCATGGTCATAGCTGTTTCCTGT	
hom-thrB-T-1	GCTATGACCATGATTACATGACCTCAGCATCT	
hom-thrB-T-2	CCCTCTAGAGTCGACGATGACGCAATG	
E-Lac promoter-1	ATATCTAGAACGCCAGCAACGCG	
E-Lac promoter-2	GTAATCATGGTCATAGCTGTTTCCTGT	
thrE-T-1	GCTATGACCATGATTACATGTTGAGTTTTGCGAC	
thrE-T-2	TATTGTCGACCGGTACCCCGGCT	
RT-*dnaE*-1	TCTCACGCCGCAGGTTAT	Primers for RT-PCR
RT-*dnaE*-2	TGTCGGCAATCAGAAAGG	
RT-*dapA*-1	TACCTACGATTTTGGCGG	
RT-*dapA*-2	GTTTGGCGTTGATTTCCC	
RT-*metX*-1	GCGATTGAAAACGACCAC	
RT-*metX*-2	CGTTCTTTTGGGCTTTGG	
RT-*hom-thrB*-1	GCCCTTTTAGGATTCGGA	
RT-*hom-thrB*-2	TACTCAATGCCGCCGATA	
RT-*thrE*-1	GTTGCCGAGAAAATCCTG	
RT-*thrE*-2	CAAACCCTTCTTTCCCAA	

into fermentation medium with 8% inoculation volume. Fermentation was carried out at 30°C, 215 rpm. for 72 h with aerobic shaking. The inducer IPTG (final concentration 0.01 mM) was added to the fermentation medium 3 hours after the initiation of fermentation.

The seed medium was (g/L): glucose 40, peptone 20, KH_2PO_4 1.5, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 5, and biotin 50 μg/L.

The fermentation medium, which was optimized by response surface methodology, was (g/L): glucose 51.60, yeast extract 6, Casein Enzymatic Hydrolysate 0.71, biotin 80 μg/L, VB_1 2 mg/L, $CaCO_3$ 20, $(NH_4)_2SO_4$ 13.87, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, $FeSO_4 \cdot 7H_2O$ 0.02, and $MnSO_4 \cdot 7H_2O$ 0.02.

2.4. Auxotroph identification of the gene knockout strains

Strains R102, R102Δ*metX*, R102Δ*dapA*, R102Δ*metX*Δ*dapA* were inoculated on MM (minimum medium), MM+Met (minimum medium supplemented with methionine), MM+Lys (minimum medium supplemented with lysine), CM (complete medium) plates, respectively, and were then incubated for 24 h at 30°C.

The complete medium was (g/L): glucose 5, yeast extract 5, peptone 10, NaCl 2.5, and agar 1.5% (w/v).

The minimum medium was (g/L): glucose 20, $(NH_4)_2SO_4$ 1.5, Urea 1.5, KH_2PO_4 1, K_2HPO_4 3, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.01, biotin 50 μg/L, and VB_1 100 μg/L. When required, the amount of amino acid added to the minimum medium was 0.15 g/L (final concentration).

2.5. Analysis of threonine by HPLC

The analysis was carried out by HPLC (Agilent 1100, USA) using the Agilent 20RBAX SB-C18 (4.6 × 150 mm, 5 μm) column with pre-column 2, 4-Dinitrofluorobenzene (DNFB) derivatization. Detection was performed by a UV detector (emission 360 nm). Separation was carried out at a flow rate of 1 mL/min using 0.05 M $NaCH_3COOH$ as eluent A (pH 6.5, adjusted with NaOH) and acetonitrile/water mixture (3:2, v/v) as eluent B. The following gradient was applied: Start 60% A, 6 min 60% A, 7 min 0% A, 9 min 60% A, and 10 min 60% A.

2.6. Preparation of total RNA and relative expression quantification of target genes

Total cellular RNA was extracted from cells with a Qiagen RNEasy Mini Kit (Qiagen, Germany) as described by the manufacturer. Then, the total RNA was purified using the kit (Macherey Nagel, Germany). Reverse transcription was performed with the PrimeScript®RT reagent kit (Takara, Japan) using a random primer mix according to the manufacturer's instructions. Real-time PCR was performed in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR® Premix EX-Taq™ II kit (Takara, Japan) with *dnaE* encoding DNA polymerase as an internal control.

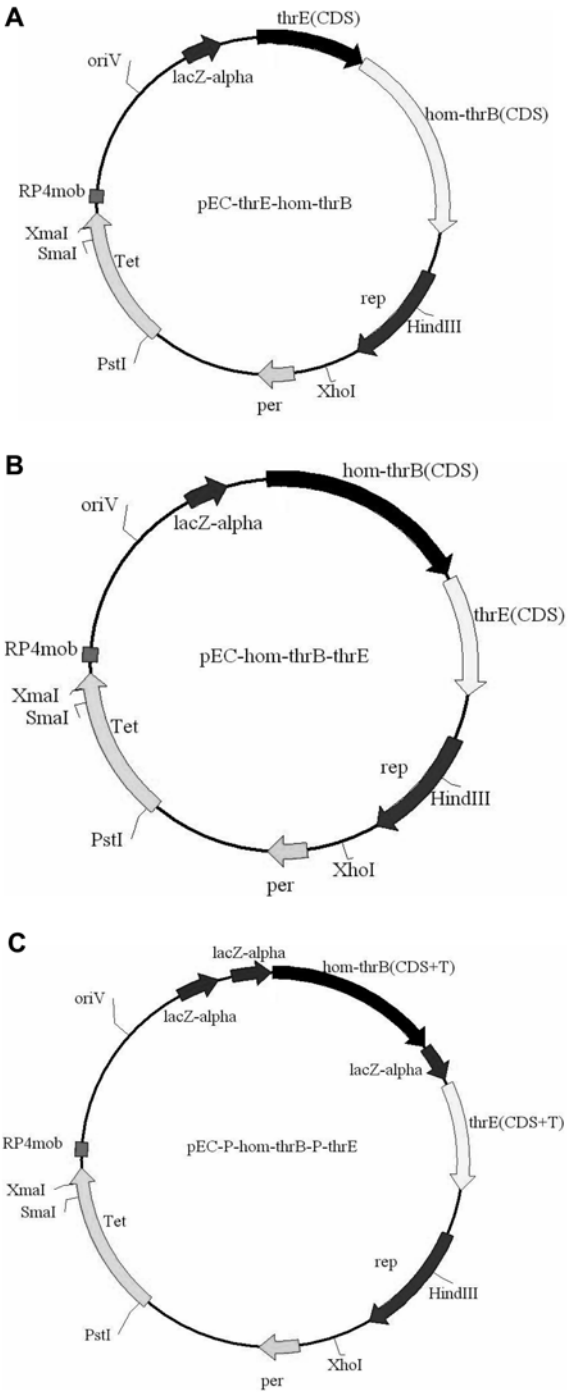

Fig. 2. Schematic diagram of the co-over-expression vectors. (A) Plasmid pEC-thrE-hom-thrB (abbreviation, pEC-E-O). (B) Plasmid pEC-hom-thrB-thrE (abbreviation, pEC-O-E). (C) Plasmid pEC-p-hom-thrB-p-thrE (abbreviation, pEC-Box).

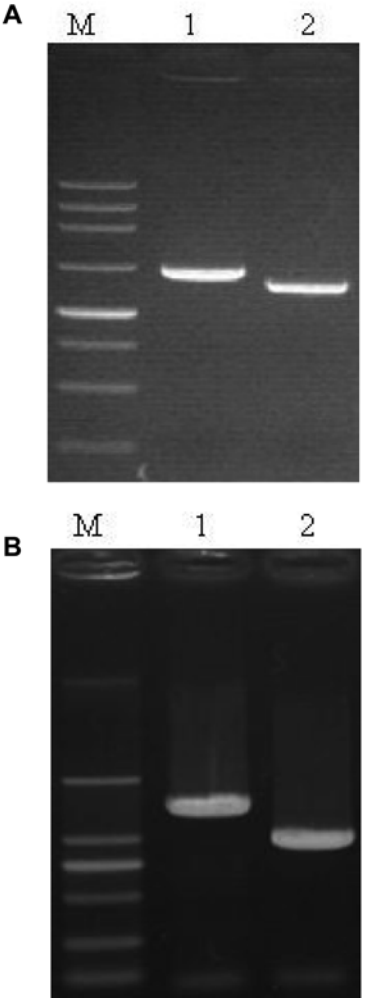

Fig. 3. Verification for deficient mutant strains with Δ*metX* or Δ*dapA*. (A) lane 1- PCR for *MetX*; lane 2- PCR for deficient mutant Δ*metX*. (B) lane 1- PCR for *DapA*; lane 2- PCR for deficient mutant Δ*dapA*.

3. Results and Discussion

3.1. Auxotrophic identification of the recombinant strains

The WT strain R102 could grow on an MM plate, while strains with Δ*metX* grew only on methionine supplemented plates which showed that strains without *metX* were definitely methionine auxotrophic. This was also confirmed by the PCR result (Fig. 3A). However, strains with Δ*dapA* demonstrated a different growth characteristic. They could grow on MM or methionine supplemented plates quite slowly after about 20 h cultivation compared to the R102. Integrated with the PCR analysis results (Fig. 3B), this implied that the gene *dapA* was successfully modified, but the lysine biosynthesis was not totally disrupted, a result which was similar to the report of Hartmann [11]. In their work, strains with an interrupted lysine pathway (simultaneous deletion of the *dapC* gene and *ddh* gene) could be successfully constructed because of the possible existence of some unknown pathway in *C. glutamicum* to synthesize *meso L-L-α,ε*-diaminopimelate, the precursor of lysine.

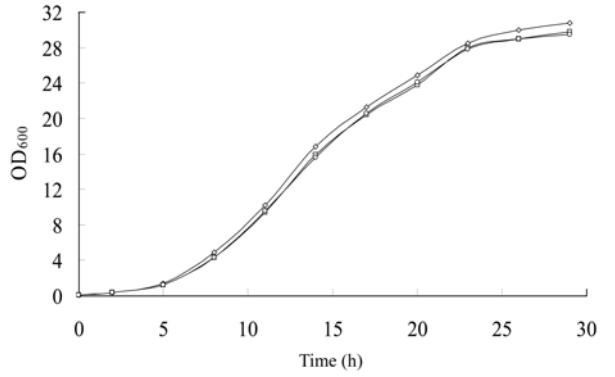

Fig. 4. Growth curves of the three strains. *Diamond* symbols R102, *square* symbols R102ΔΔ, *circle* symbols R102ΔΔ (pEC-Box).

3.2. Growth curves of the three strains

Growth curves of seed cultivation at 30°C, 250 rpm. were as follows (Fig. 4).

Compared with the WT strain R102, the two recombinant strains grew slowly at the beginning but finally the biomass was approximate. We could also conclude that the deletion of the genes had little influence on strain growth, which was favorable for scaled-up fermentation.

3.3. Evaluation of threonine yield and target gene relative expression quantification of the recombinant strains

Fig. 5 indicates that the threonine yield increased from 1.80 g threonine/L to 3.35 g threonine/L by the systematic genetic construction. Inactivation of *metX* and *dapA* could lead to a remarkable increase in threonine yield compared with R102 (from 1.80 g threonine/L to 2.58, 2.38 and 3.01 g threonine/L, increased by 42.5, 31.5, and 66.3% respectively), qPCR results showed a dramatic decrease in relative expression quantification of *metX*, *dapA* genes (Fig. 6). Although the modification of *dapA* gene did not totally block the lysine biosynthetic pathway as mentioned above, it still demonstrated a relatively positive impact on threonine yield, which indicated the importance of increasing the precursors supply in threonine production.

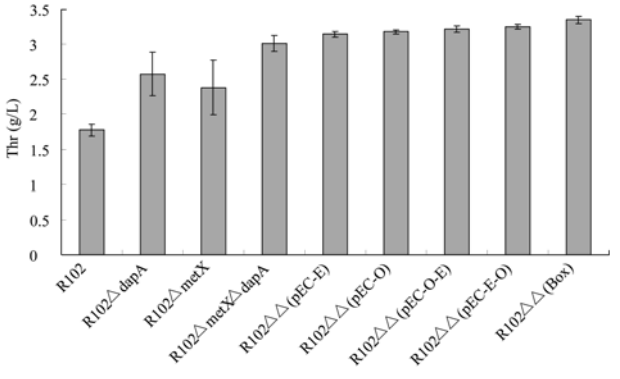

Fig. 5. Threonine yield of the relative strains.

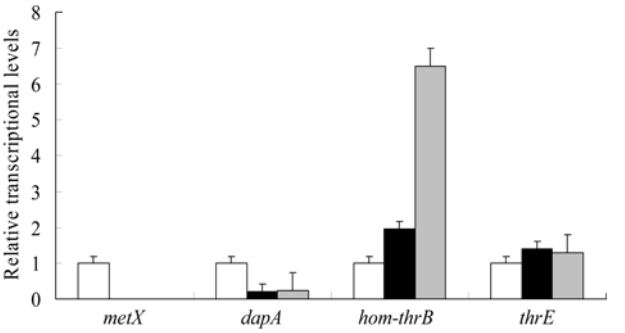

Fig. 6. Changes in the transcript levels of genes involved in our research during aerobic cultivation. *White* symbols R102, *black* symbols R102ΔΔ (pEC-O-E), *gray* symbols R102ΔΔ (pEC-Box).

Overexpression of hom_{R102}-*thrB* and *thrE* respectively or coherently in the recombinant strain R102Δ*metX*Δ*dapA* could lead to a further increase of the threonine yield, but the increased slope was less than that of the gene knockout (from 3.01 g threonine/L to 3.14, 3.18, 3.21, 3.24, and 3.35 g threonine/L, increased by 4.3,5.6,6.6,7.6, and 11.3% respectively).

Different initial strains could lead to significantly different responses to the operon over-expression. The mutated threonine operon hom_{FBR}-*thrB* was reported to be over-expressed in the lysine-plus-threonine producer *C. glutamicum* DM 368-3 [12], which led to a notable increase in threonine yield, from 6.3 (0.75 g threonine/L) to 14.2 mM (1.69 g threonine/L). Amplification of native threonine operon *hom-thrB* in *C. glutamicum* DG52-5 could also increase threonine yield, while in the *C. glutamicum* ATCC13032 it did not result in any secretion of threonine.

The sequence variation of gene hom_{R102} (a single nucleotide deletion in codon 429 within the *hom* reading frame) [6], but in *C. glutamicum* DM 368-3 (a single transition from G to A in hom_{FBR} leading to replacement of glycine-378 by glutamate) might also contribute to the response divergency.

The effect of *thrE* on threonine production was also not very impressive in other works [5,13] because of the relative low exporter activity. The problem might be solved by using these exporters in *E. coli* [13] or some other exporters such as *lysE* for lysine and arginine [14].

There was no significant difference in threonine yield between the different co-expression methods, as shown in Fig. 5. The relatively better result from R102ΔΔ(pEC-Box) might be explained by the data from qPCR. It indicated that the hom_{R102}-*thrB* and *thrE* gene cluster, under control of one lac promoter (Figs. 2A and 2B), showed a slight increase in gene transcription level (about 1.5 fold); while under control of the respective lac promoter (Fig. 2C), the relative expression quantition of *hom-thrB* was dramatically higher than the former (about 6 fold), but the transcription level of *thrE* was still nearly the same as the former

(about 1.5 fold). The qPCR results suggested that the enhanced transcription level of hom_{R102}-*thrB* genes might result in a better threonine production capacity of recombinant strain R102ΔΔ(pEC-Box). The relative low transcription level of *thrE* might also hamper its function. Although the plasmid diagram was not mentioned [13], a similar transcription profile was observed.

A further evaluation of the systematic strategy for threonine production in *Corynebacterium* needs to be studied from different aspects, including the translation level of the key enzyme, metabolic flow and intermediate product accumulation profile.

4. Conclusion

Because of their endotoxins, *E. coli* strains are not appropriate for pharmaceutical-grade amino acid production [1]. Additionally, *C. glutamicum* has fewer isoenzymes; therefore its biosynthesis pathway is more effective than that in *E. coli.* In our research, a systematic modification of the threonine biosynthesis pathway in *C. glutamicum* R102 was manipulated accordingly. Recombinant strains R102Δ*metX*, R102Δ*dapA*, R102Δ*metX*Δ*dapA* were obtained for improved threonine yield by gene knockout instead of random mutation. There was a further increase in threonine yield by additional over-expression of *hom-thrB* and *thrE*. Further target genes could be identified by practical experiments combined with *in silico* simulation [15]. The overall threonine yield was finally increased by 86.1%, from 1.80 to 3.35 g threonine/L. With further optimization of strain construction strategy and system biology applied in *Corynebacterium glutamicum* [16], the food-safety-grade *C. glutamicum* strains are prospective as industrial L-threonine producers in the future.

Acknowledgements

We appreciate the assistance of A. J. Sinskey, Chris Brigham, and Laura B. Willis for providing strain R102 and some useful methods in our studies. We also appreciated Andreas Tauch for providing cloning vectors pK18mob2 and pEC-T18mob2. The authors gratefully acknowledge the financial support from the Fundamental Research Funds for the Central Universities, SCUT, project No. 2009ZM0289.

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