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Research Article

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Modification of L-threonine producing *Escherichia coli* for L-isoleucine production

Bing Wen[3,4], Haibin Zhang[3], Yuan Liu[3], Na Wei[4], Chenglin Zhang[1,2,3], Qingyang Xu[1,2,3] and Ning Chen[1,2,3*]

[1]*Key Laboratory of Industrial Microbiology, Education Ministry, Tianjin University of Science and Technology, Tianjin, China*
[2]*National and Local United Engineering Lab of Metabolic Control Fermentation Technology, Tianjin, China*
[3]*College of Biotechnology, Tianjin University of Science and Technology, Tianjin, China*
[4]*Tianjin Research Institute of Industrial Microbiology, Tianjin, China*
Bing We and Haibin Zhang are Co-first authors

ABSTRACT

As an essential amino acid, L-isoleucine can improve endurance and assists in the repair and rebuilding of muscle and serve as a major supplement in infusions and special diets. So far, Corynebacterium glutamicum is commonly used for the industrial production of L-isoleucine, however, period of L-isoleucine fermentation by the bacterium is relative long. In this study, we aimed at constructing a genetically defined E. coli strain capable of producing L-isoleucine using L-threonine-producing THRD as a starting strain. It was modified by co-overexpressing desensitized threonine dehydratase (encoded by ilvA) and acetohydroxy acid synthase III (encoded by ilvIH) to further increase the flux from L-threonine to L-isoleucine. The final engineered strain was able to produce 1.54 g/L of L-isoleucine by shake flask fermentation. The design principles described in this study would be useful to construct strains for producing other similar biological products.

Keywords: L-threonine, L-isoleucine, threonine dehydratase, acetohydroxy acid synthase, feedback inhibition

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INTRODUCTION

Amino acids are important products that have been used in food, pharmaceutical, agriculture and cosmetic industries[1]. As one of essential amino acids, L-isoleucine can improve endurance and assists in the repair and rebuilding of muscle, and it serves as a major supplement in infusions and special diets[2]. At present, *Corynebacterium glutamicum* is commonly used for the industrial production of L-isoleucine[3]. However, period of L-isoleucine fermentation by *C. glutamicum* is relative longer.

Escherichia coli has been widely used for L-threonine, L-tryptophan, and L-phenylalanine[4-6]. Since L-threonine is one of precursor for L-isoleucine synthesis and the fermentation period by *E. coli* is rather shorter, it made sense to modify L-threonine producing *E. coli* for L-isoleucine production. In *E. coli*, L-isoleucine is synthesized through five enzymatic reactions from L-threonine as a precursor. As showed in Figure 1, L-isoleucine is synthesized from the condensation of 2-ketobutyrate and pyruvate catalyzed by acetohydroxy acid synthase (AHAS, EC 2.2.1.6), and 2-ketobutyrate is formed from L-threonine by threonine deaminase(TD, EC 4.2.1.16). It is discovered that TD (encoded by *ilvA*) involved in the first limiting step toward L-isoleucine synthesis and the activity of TD is inhibited by L-isoleucine[7]. The reaction catalyzed by AHAS is the secondary limiting step, however, there are three isozymes of AHAS(AHAS I, AHAS II, AHAS III, encoded by *ilvBN*, *ilvGM*, and *ilvIH*, respectively) in *E. coli*[8]. And it is reported that AHAS II and AHAS III have much higher affinity to 2-ketobutyrate than pyruvate, however, AHAS II is not expressed due to the frameshift muptation in *ilvG* in *E. coli*[8]. So the AHAS III seems to be more

suited for L-isoleucine biosynthesis[9, 10]. The genes for TD, AHAS III, dihydroxyacid dehydratase(DH, encoded by *ilvD*, EC 4.2.1.9) and branched-chain amino-acid aminotransferase (encoded by *ilvE*, EC 2.6.1.42) constitute the *ilvGMEDA* operon, and its expression is controlled by mutivarent attenuation by L-isoleucine, L-vanline, and L-leucine[8, 11]. In addition, since *ilvA* located at downstream of the operon, its transcription level seems rather low.

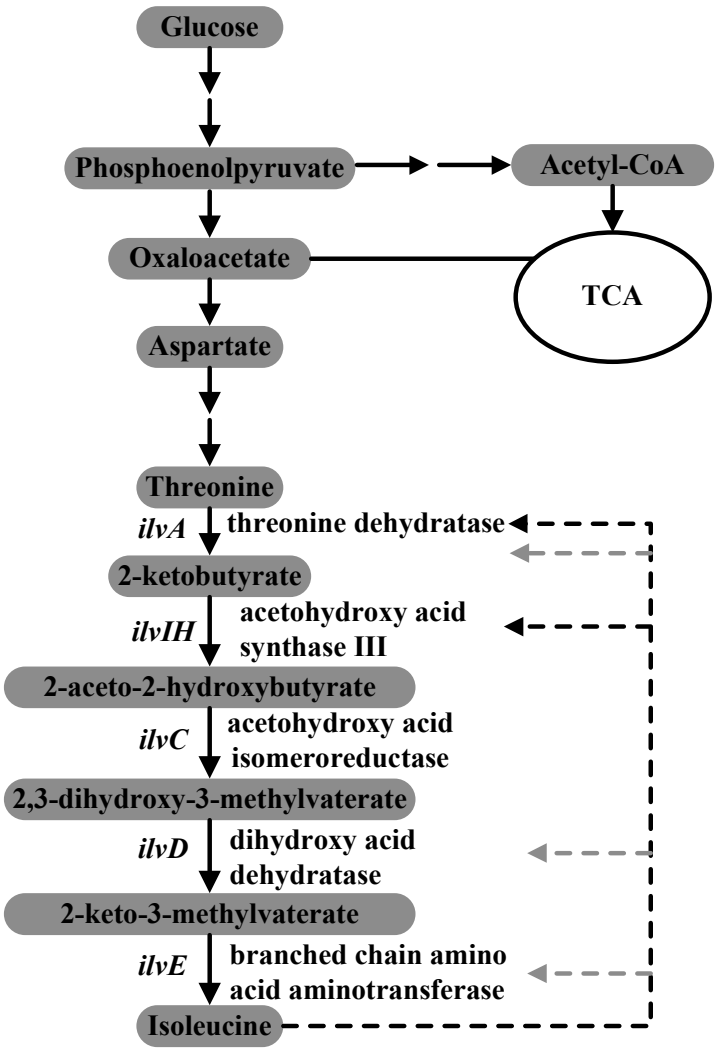

Fig. 1 Biosynthetic pathway of L-isoleucine involved in *E. coli*. Black dotted lines indicate feedback inhibition. Gray dotted lines indicate transcriptional attenuation regulation

In this study, desensitized *ilvA* and *ilvIH* was overexpressed in L-threonine producing strain THRD to investigate potential effects of the genes on L-isoleucine production.

EXPERIMENTAL SECTION

2.1 Strains, Plasmids and Primers

The strains and plasmids used in this study are listed in Table 1. The primers are listed in Table 2. All DNA manipulations were carried out by following standard protocols.

Table 1 Strains and plasmids

Name	Characteristics	Source
Strains		
E. coli THRD	L-threonine producer (ILE^L, AHV^r)	Our lab
THRD-A	THRD haboring pWSK29-ilvA	This study
THRD-AIH	THRD haboring pWSK29-ilvAIH	This study
Plasmid		
pWSK29	Low copy vector, Amp^r	Our lab
pWSK29-ilvA	Mutant *ilvA* cloned in the *Xba* I and *Bam*H I site of pWSK29	This study
pWSK29-ilvAIH	Mutant *ilvIH* cloned in the *Pst* I and *Xho* I site of pWSK29-ilvA	This study

Table 2 Primers

Name	Sequence (5'-3')*
ilvA-1	*TCTAGA*AAAGAAGGAGATATACAATGGCTGACTCGCAACCC (*Xba* I)
ilvA-2	CAGCGTGTTGGCGAAGCGCAGAAACGCGCCCG
ilvA-3	CGGGCGCGTTTCTGCGCTTCGCCAACACGCTG
ilvA-4	*GGATCC*CTAACCCGCCAAAAAGAACC (*Bam*H I)
ilvH-1	*CTGCAT*GGTTAATGTGTTTTACACATTTTTTCCG (*Pst* I)
ilvH-2	GGAAAAAAGGCCAATCACGCGGAATAACGCGTCTGATTCATTTTCGAGTA
ilvH-3	TACTCGAAAATGAATCAGACGCGTTATTCCGCGTGATTGGCCTTTTTTCC
ilvH-4	*CTCGAG*TCAACGCATTATTTTATCGCCG (*Xho* I)

** Mutated bases are in underline and restriction sites are in italic.*

2.2 Media and culture conditions

Luria-Bertani(LB), Super Optimal Broth(SOB), and Super Optimal broth with Catabolite repression(SOC) media were used for culturing *E. coli* cells(grown at 37 °C). Seed medium contained 30 g sucrose, 10 g yeast extract, 10 g $KH_2PO_4 \cdot 3H_2O$, 10 g $(NH_4)_2SO_4$, and 0.6 g $MgSO_4 \cdot 7H_2O$ per liter. Fermentation medium contained 35 g glucose, 12 g yeast extract, 15 ml corn steep liquor, 8 g $KH_2PO_4 \cdot 3H_2O$, 10 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.01 g $MnSO_4 \cdot H_2O$ per liter.

2.3 Site-Directed of *ilvA* and *ilvIH* and construction of recombinant plasmid

Primers ilvA-1 and ilvA-2, containing four mutated bases (1339^{th} C→T, 1341^{st} G→T, 1351^{st} C→G, 1352^{nd} T→C), were used to amplify a 1362-bp DNA fragment using the genomic DNA of *E. coli* MG1655 as a template. Another DNA fragment of 215-bp was amplified using the primers ilvA-3, containing four mutated bases (1339^{th} G→A, 1341^{st} C→A, 1351^{st} G→C, 1352^{nd} A→G), and ilvA4. Two DNA fragments were purified and mixed, and the complete 1561-bp fragment was amplified by overlapping PCR using the primers ilvA1 and ilvA4. The resulting fragment was digested with *Xba* I and *Bam*H I and ligated into the *Xba* I- *Bam*H I-digested pWSK 29, resulting in pWSK-ilvA. Primers ilvIH-1 and ilvIH-2 containing two mutated bases (1768^{th} G→A, 1777^{th} C→T) were used to amplify a 1796-bp DNA fragment using the genomic DNA of *E. coli* MG1655 as a template. Another DNA fragment of 470-bp was amplified with the primers ilvIH-3 containing two mutated bases (1768^{th} C →T, 1777^{th} G→A), and ilvIH-4 using the same template. Two DNA fragments were purified and mixed, and the complete 2266-bp fragment was amplified by overlapping PCR using the primers ilvIH-1 and ilvIH-4. The fragment was digested with *Pst* I and *Xho* I and was ligated into the *Pst* I-*Xho* I-digested pWSK-ilvA, resulting in pWSK-ilvAIH. Successful substitution of the bases was confirmed by sequencing.

PWSK-ilvA and pWSK-ilvAIH were transformed into L-threonine producing strain *E. coli* THRD, resulting in THRD-ilvA and THRD-ilvAIH respectively.

2.4 Fermentation

Shake flask fermentation was carried out by adding 40 ml of seed culture to a 500-ml shake flask, and was incubated at 37° C by rotating at 200 rpm. Then 4 ml of seed culture (OD_{600}= ~10) was inoculated into 26 ml fermentation medium in a 500-ml shake flask. During fermentation process, pH was maintained at about 7.0 by adding of ammonia.

2.5 Analytics

During the fermentation, fermentation liquor of 1 ml were taken from the cultures and centrifuged at 4°C and 10,000 g for 5 min. For biomass determination, cell dry weight was determined gravimetrically. Glucose was determined with biosensor (Institute of Biology, Shandong Academy of Science). 2-ketobutyrate and L-isoleucine was analyzed by using high performance liquid chromatography (HPLC).

2.6 Statistical analysis

All experiments were conducted in triplicates and data were averaged and presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were used to determine significant differences. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

3.1 Effect of overexpression of *ilvA* on L-isoleucine production

In the theronine-producing strain *E. coli* THRD, feedback inhibitions of phosphoenolpyruvate carboxylase and aspartokinase I by L-aspartate and L-threonine were removed, respectively. And the transcriptional attenuator leader region of the *thrABC* operon was deleted. Since L-isoleucine is synthesized through five enzymatic steps from L-threonine, THRD strain was used for constructing the L-isoleucine production strain. Further rational metabolic

engineering was performed to develop an L-isoleucine producing strain.

TD (encoded by *ilvA*) catalyzing the first step in L-isoleucine biosynthesis was feedback inhibited by L-isoleucine. It was reported that feedback inhibition of threonine dehydratase could be removed by replacing the 1339th base C with T, 1341th G with T, 1351th C with G and 1352th T with C[12, 13]. So the mutant *ilvA*, encoding the feedback-resistant threonine dehydratase, was cloned into pWSK29 to make pWSK-ilvA. Then the recombinant pWSK-ilvA was transformed into THRD. Fermentation assay with THRD-A was performed to detect the effect of *ilvA* overexpression on L-isoleucine production. Result showed that THRD-A could successfully produce 0.22 g/L of L-isoleucine(Table 3). Notably, 9.72 g/L of 2-ketobutyrate was produced by THRD-A but almost none L-threonine was detected in the fermentation liquor, which indicated that overexpressed threonine dehydratase effectively catalyzed threonine to 2-ketobutyrate and accumulation of 2-ketobutyrate was probably due to low speed of L-isoleucine synthesis from it.

Effect of co-overexpression of *ilvA* and *ilvIH* on L-isoleucine production

E. coli possesses three of AHAS's, differing in biochemical properties and regulation mechanisms. Among the three isoenzymes, AHAS III, encoded by *ilvIH*, has a much higher affinity for 2-ketobutyrate and AHAS II is not expressed due to the frameshift mutation in *ilvG*. Therefore *ilvIH* was selected for amplification to enhance consumption of 2-ketobutyrate.

However, activity of AHAS III was feedback inhibited by both L-isoleucine and L-valine and it was reported that the inhibition could be removed by replacing the 41st G with A and 50th C with T[10, 14]. The mutant *ilvIH* was gained by overlapping PCR and was cloned to down stream of *ilvA* in pWSK29ilvA, resulting pWSK-ilvAIH. Then the recombinant plasmid was transformed into THRD to construct THRD-AIH.

Fermentation was performed to detected effect of co-expressing *ilvA* and *ilvIH* on L-isoleucine production by THRD-AIH. Result showed that THRD-AIH could successfully produce 1.54 g/L of L-isoleucine. Notably, accumulation of 2-ketobutyrate decreased to only 1.32 g/L and none threonine was detected in the fermentation liquor(Table 3).

Table 3 Concentration of L-isoleucine, L-threonine and 2-ketobutyrate accumulated by THRD-A and THRD-AIH

Strains	Concentration (g/L)		
	L-isoleucine	L-threonine	2-ketobutyrate
THRD-A	0.22±0.01	0.05±0.01	9.72±0.32
THRD-AIH	1.54±0.02	0.03±0.01	1.32±0.07

L-isoleucine has been usually manufactured by bacterial fermentation, mainly employing mutant strains of *C. glutamicum* and the annual production was about 400 tons. In the recent years, various uses of L-isoleucine have been explored: components of cosmetics and pharmaceuticals, animal feed additives, additives in infusion solutions and dietary products, and precursors in the chemical synthesis of herbicides. So the demand for L-isoleucine is increasing. In this study, we aimed at constructing a genetically defined *E. coli* strain capable of producing L-isoleucine. Since L-isoleucine was synthesized from L-threonine through five reactions, the L-threonine-producing strain, *E. coli* THRD, was used as a starting strain. It was modified by following strategies as removal of feedback inhibitions of threonine dehydratase and AHAS III. Even though L-isoleucine production by THRD-AIH was rather low, this is the only the first step of developing an industrially applicable strain. Further metabolic engineering strategies such as amplification of *ilvC*, enhancement of L-isoleucine exporter (YgaZH) expression, deletion of L-isoleucine carrier encoding gene *brnQ* and deletion of transcription repressor encoding gene *iclR* should be taken to enhance L-isoleucine production.

CONCLUSION

Overexpression of *ilvA* could increase metabolization of threonine to 2-ketobutyrate and lead to accumulation of 2-ketobutyrate. Co-overexpression of desensitized threonine dehydratase and acetohydroxy acid synthase III could increase the flux from L-threonine to L-isoleucine. The design principles described in this study would be useful to construct strains for producing other similar biological products.

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