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

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**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

**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 mutation 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 \textit{ilvD}, EC 4.2.1.9) and branched-chain amino-acid aminotransferase (encoded by \textit{ilvE}, EC 2.6.1.42) constitute the \textit{ilvGMEDA} operon, and its expression is controlled by multivarent attenuation by L-isoleucine, L-vanline, and L-leucine[8, 11]. In addition, since \textit{ilvA} located at downstream of the operon, its transcription level seems rather low.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig1}
\caption{Biosynthetic pathway of L-isoleucine involved in \textit{E. coli}. Black dotted lines indicate feedback inhibition. Gray dotted lines indicate transcriptional attenuation regulation.}
\end{figure}

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

\section*{EXPERIMENTAL SECTION}

\subsection*{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.

\begin{table}[h]
\centering
\caption{Strains and plasmids}
\begin{tabular}{|l|l|l|}
\hline
\textbf{Name} & \textbf{Characteristics} & \textbf{Source} \\
\hline
\textit{Strains} &  &  \\
\textit{E. coli} THRD & L-threonine producer (ILE\textsuperscript{L}, AHV\textsuperscript{r}) & Our lab \\
THRD-A & THRD haboring pWSK29-ilvA & This study \\
THRD-AIH & THRD haboring pWSK29-ilvAIH & This study \\
\textit{Plasmid} &  &  \\
pWSK29 & Low copy vector, Amp\textsuperscript{r} & Our lab \\
pWSK29-ilvA & Mutant \textit{ilvA} cloned in the Xho I and BamH I site of pWSK29 & This study \\
pWSK29-ilvAIH & Mutant \textit{ilvIH} cloned in the Pst I and Xho I site of pWSK29-ilvA & This study \\
\hline
\end{tabular}
\end{table}
with biosensor (Institute of Biology, Shandong Academy of Science). 2-ketobutyrate and L-isoleucine was analyzed for 5 min. For biomass determination, cell dry weight was determined gravimetrically. Glucose was determined during the fermentation, fermentation liquor of 1 ml were taken from the cultures and centrifuged at 4 °C and 10,000 g for 10 min.

2.5 Analytics

Ammonia was determined using high performance liquid chromatography (HP LC).

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 threonine-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 treatments were employed to increase the L-isoleucine production.

Table 2 Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ilvA-1</td>
<td>TCTAGAAGAGAAGCATATCAGCTGACTTCGCAACCC (Xho I)</td>
</tr>
<tr>
<td>ilvA-2</td>
<td>CAGCGTGTTGGCGAAGCGCAGAAAGCGGCCGCG</td>
</tr>
<tr>
<td>ilvA-3</td>
<td>CGGCGGCGGTTCCTGCCTCCGCAACACCGCTG</td>
</tr>
<tr>
<td>ilvA-4</td>
<td>GGATCCCTAACCCGCCAAAAGAACC (BamH I)</td>
</tr>
<tr>
<td>ilvH-1</td>
<td>CTGACGTTTAAGTTGTTCATACATTTTTCGG (Pst I)</td>
</tr>
<tr>
<td>ilvH-2</td>
<td>GGAAAAAAGGCAATACCGCGGATACGGCGCTGATTCAATTTCGGA</td>
</tr>
<tr>
<td>ilvH-3</td>
<td>TACCTCAGAAAATGTAAGCAGCCTGATCCGATGTCGGCTTTTTCG</td>
</tr>
<tr>
<td>ilvH-4</td>
<td>CTCGAGTCAAAGCCATATGTATATCGCCG (Xho I)</td>
</tr>
</tbody>
</table>

* Mutated bases are in underline and restriction sites are in italic.
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 41\[^{st}\] G with A and 50\[^{th}\] C with T\[^{[10, 14]}\]. The mutant \( ilvIH \) was gained by overlapping PCR and was cloned to down stream of \( ilvA \) in pWSK29-ilvA, 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**

<table>
<thead>
<tr>
<th>Strains</th>
<th>L-isoleucine (g/L)</th>
<th>L-threonine (g/L)</th>
<th>2-ketobutyrate (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THRD-A</td>
<td>0.22±0.01</td>
<td>0.05±0.01</td>
<td>9.72±0.32</td>
</tr>
<tr>
<td>THRD-AIH</td>
<td>1.54±0.02</td>
<td>0.03±0.01</td>
<td>1.32±0.07</td>
</tr>
</tbody>
</table>

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.

**Acknowledgments**

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