Note

Improvement of Reticuline Productivity from Dopamine by Using Engineered Escherichia coli

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Benzylisoquinoline alkaloids (BIAs) are pharmaceutically important compounds. We have previously devised a reticuline (BIA) production method from dopamine by using Escherichia coli; however, its productivity was relatively low (33 μM, 11 mg/L). We report here, by fine-tuning the method, higher reticuline productivity of 165 μM (54 mg/L), increasing the conversion efficiency by 8-fold. These results are important for developing an efficient route to fermentative reticuline production.

Key words: benzylisoquinoline alkaloid; reticuline; (S)-norcoclaurine synthase; monoamine oxidase; microbial production

Benzylisoquinoline alkaloids (BIAs) are a large and diverse group of secondary metabolites synthesized by plants in the Papaveraceae, Berberidaceae, Ranunculaceae, and Magnoliaceae families.† BIAs have been shown to have various pharmacological effects, including analgesic properties (morphine), antibacterial activity (berberine), and antioxidative activity (magnolol).‡ There is a strong demand for BIAs, but they are currently mainly obtained by direct extraction from plants, which is a low-yield process. Several chemical methods have been developed to synthesize BIAs, but their costs are prohibitive because multi-step reactions are required to synthesize the complex molecular structure of these compounds. Some plant biotechnological strategies have been developed equally to produce specific BIAs; however, their practical use has been limited because of the difficulty in controlling the biosynthetic pathway in plants.

Microbial production is one of the strategies to obtain BIAs. Although it involves unavoidable problems related to the expression of heterologous enzymes, secondary metabolite production in microbes is generally highly efficient because of its easy handling and short incubation period.§ In addition, microbial produc-

Fig. 1. Artificial Reticuline Production Pathway from Dopamine in an Engineered Strain of E. coli.

MAO converts dopamine to 3,4-DHPAA which is condensed with dopamine by NCS to produce THP. THP is then converted to reticuline through three-step methylation.§

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¶ Abbreviations: 3,4-DHPAA, 3,4-dihydroxyphenylacetaldehyde; MTs, methyltransferases (6-O-methyltransferase, coclaurine-N-methyltransferase and 4’-O-methyltransferase) in the reticuline synthetic pathway; BIA, benzylisoquinoline alkaloid; IPTG, isopropyl β-D-thiogalactopyranoside; MAO, monoamine oxidase; NCS, (S)-norcoclaurine synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THP, tetrahydrodipapaverol

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ing tetrahydropapaveroline (THP) (Fig. 1). THP is then converted to reticuline by three sequential methylation steps.4,5 An Escherichia coli strain harboring this biosynthetic pathway has successfully produced reticuline from dopamine, although its conversion efficiency was less than 1.3%: 33 μM (11 mg/L) of reticuline was produced from 5 mM dopamine (note that one molecule of reticuline was synthesized from two molecules of dopamine).3 We therefore attempted in this present study to improve the productivity of the method by fine-tuning the possible rate-limiting step in the reactions.

We first focused on the competition between MAO and NCS for dopamine, since dopamine was the substrate for both of these enzymes. Indeed, an in vitro study using purified MAO and NCS revealed the molar ratio between the enzymes to be a critical factor in THP production (data not shown). To address this limitation, NCS from Copris japonica (GenBank accession no. AB267399) and MAO (codon-optimized) from Micrococcus luteus (GenBank accession no. AB010716) were introduced into E. coli by using two vectors with the different copy numbers, pMW118 and pCDFPL. pMW118 (Nippon Gene Co.) has a pSC101 origin and its copy number is approximately five per cell.7) pCDFPL, which was constructed in this study, carries the CloDF13 replicon, whose copy number per cell is less than 1.3%: 33 μM (11 mg/L) of reticuline was produced from 1 mM dopamine when the OD₆₀₀ value had reached 0.5. The amount of reticuline in the culture medium was analyzed by LC-MS as previously described.3) The strain harboring NCS in pCDFPL showed higher reticuline productivity than that of the strain harboring NCS in pMW118. In contrast, different copy numbers of MAO did not significantly affect the reticuline productivity (Fig. 2A). These data suggested that the amount of NCS was more critical than that of MAO for reticuline production. The strain carrying pCDFPL-MAO-NCS showed the highest productivity among the tested strains: the yield was 41 μM reticuline from 1 mM dopamine, a 14-fold higher conversion efficiency than that obtained by using the previously constructed strain (2.9 μM reticuline from 1 mM dopamine).3) We next optimized the temperature to further improve the productivity of the system, and found that 20 °C was optimal for reticuline production (Fig. 2B). Under the conditions used here, the maximum productivity was 88 μM reticuline from 1 mM dopamine. The productivity was markedly reduced at 30 °C, and no reticuline could be detected at 37 °C. The intermediates for reticuline had a catechol ring that was unstable at high temperatures, making lower temperatures more suitable for reticuline production. Increasing the amount of dopamine did not significantly improve reticuline production (Fig. 2C). The pH value was also an important factor affecting productivity, because dopamine, 3,4-DHPAA, and THP were readily oxidized at alkaline pH values. We analyzed the effect of pH on reticuline production by adjusting the pH value of the culture medium with 70 mM potassium phosphate. We found pH 6.0 to be optimal for reticuline production. A pH value less than 6.0 was deleterious to E. coli cells. The reticuline productivity decreased with increasing pH value, probably reflecting the oxidation of dopamine and other intermediates on the synthetic pathway. Indeed, the dopamine concentration immediately decreased when cells were grown under alkaline conditions (data not shown). The strain produced 165 μM reticuline from 3 mM dopamine under the optimized conditions (Fig. 2D). The conversion efficiency from dopamine to reticuline was therefore 8-fold higher than that by the previous method (1.3% vs. 11%), and the productivity was 5-fold higher (33 μM vs. 165 μM).

### Table 1. Plasmids and Primers Used in This Study

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>5' primer*</th>
<th>3' primer*</th>
<th>Description</th>
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<tbody>
<tr>
<td>pCDFPL-NCS</td>
<td>5'-GGGGACGTCGAAA TTAATCGACTGACTATA3'</td>
<td>5'-GGGCTCGAGTTAT TCGAGATTGTTGTTT-3'</td>
<td>NCS with T7 promoter fragment was amplified from pET21d-NCS.7)</td>
</tr>
<tr>
<td>pMW118-NCS</td>
<td>5'-CCCGAATTCCAAT TTAATCGACTGACTATA3'</td>
<td>5'-GGGCTCGAGTTAT TCGAGATTGTTGTTT-3'</td>
<td>NCS with T7 promoter fragment was amplified from pET21d-NCS.7)</td>
</tr>
<tr>
<td>pCDFPL-MAO</td>
<td>5'-AAAGGGCGGCGAAA TTAATCGACTGACTATA3'</td>
<td>5'-AAAGCGCTCTAA GACGTTACAGGCG-3'</td>
<td>The codon-optimized MAO gene with T7 promoter was amplified from pGS21a-optMAO.7)</td>
</tr>
<tr>
<td>pMW118-MAO</td>
<td>5'-CCCGAATTCCAAT TTAATCGACTGACTATA3'</td>
<td>5'-TTTGGATCCCTTAA TGCGCCGCTACAGGGC-3'</td>
<td>The codon-optimized MAO gene with T7 promoter was amplified from pET21d-NCS.7)</td>
</tr>
<tr>
<td>pCDFPL-MAO-NCS</td>
<td>5'-GGGGACGTCGAAA TTAATCGACTGACTATA3'</td>
<td>5'-GGGCTCGAGTTAT TCGAGATTGTTGTTT-3'</td>
<td>NCS with T7 promoter was amplified from pET21d-NCS.7) and cloned into the cognate sites of pCDFPL-MAO.</td>
</tr>
<tr>
<td>pMW118-MAO-NCS</td>
<td>5'-GGGCTCGAGAAA TTAATCGACTGACTATA3'</td>
<td>5'-GGGCTCGAGTTAT TCGAGATTGTTGTTT-3'</td>
<td>NCS with T7 promoter was amplified from pMW118-MAO-NCS.7) and cloned into XhoI site of pMW118-MAO.</td>
</tr>
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*Restriction sites are underlined.
We have demonstrated here that increasing the NCS expression level and stabilizing the intermediates on the reticuline synthetic pathway were important factors for improving reticuline production. Among all of our current and previously described reticuline-producing strains,3,9,10) the strain constructed in this study (pCDFPL-NCS-MAO) showed the highest productivity per cell (38 \mu M/OD_{600}) when cells were cultured at 20 °C at pH 6.0. However, the culture medium was still black because of the accumulation of oxidized intermediates that formed a melanin-like pigment. We have tried to construct a fermentative system for reticuline production by using glucose as the starting material to circumvent this problem. The results presented in this note provide important clues to further improving the fermentative system.

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References