Bench-top fermentative production of plant benzylisoquinoline alkaloids using a bacterial platform

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The plant secondary metabolites benzylisoquinoline alkaloids (BIAs) have diverse pharmaceutical activities, and some are used medicinally (e.g., morphine, codeine, berberine). Recently, we constructed a platform to produce BIAs using bioengineered Escherichia coli, which could be useful for bulk production. The E. coli strain used in this system produces the important intermediate (S)-reticuline from glucose or glycerol. Although the amount produced (40 mg/L) exceeded the amount that can be purified from plants, the conversion efficiency from glycerol was only 0.15%; thus, there was much room for improvement. Our production system was developed in a jar fermenter but it is difficult to work with multiple samples using this system. In contrast, many samples can be cultured in parallel using shake flask cultures, allowing optimization of production conditions. Here, we describe bench-top production of (S)-reticuline and optimization of culture conditions using shake flask cultures. The production of (S)-reticuline reached 33.9 mg/L.

Reticuline-Producing Strain

Higher plants produce diverse chemicals such as alkaloids, terpenoids, and phenolic compounds in secondary metabolism. Among these chemicals, alkaloids are very important in medicine because of their strong biological activities. Alkaloids are low-molecular-weight, nitrogen-containing compounds found in ~20% of plant species. Benzylisoquinoline alkaloids (BIAs) are a large and diverse group of pharmaceutical alkaloids with ~2,500 defined structures. In the BIA pathway, BIAs (including the analgesic compounds morphine and codeine and the antibacterial agents berberine and palmatine) are produced via (S)-reticuline from L-tyrosine. (S)-Reticuline is a branch-point intermediate in the biosynthesis of many types of BIAs, and also a non-narcotic alkaloid of pharmaceutical significance that is useful for the development of antimalarial and anticancer drugs.

Recently, there has been increasing interest in microbial production of plant secondary metabolites by reconstructing their biosynthetic pathways in microorganisms.1–5 We previously constructed an (S)-reticuline-producing strain of Escherichia coli, which can produce (S)-reticuline from glucose or glycerol. The platform strain has three pathways: (1) an L-tyrosine over-producing pathway; (2) a dopamine-producing pathway from L-tyrosine; and (3) an (S)-reticuline-producing pathway from dopamine6 (Fig. 1). The L-tyrosine over-producing strain was constructed by modifying the E. coli BL21(DE3) strain. It has a disrupted tyrR gene, which encodes a repressor of several genes related to the shikimic acid pathway,7 and was modified by introduction of four overexpressed enzymes: feedback resistant (fbr) 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (fbr-DAHPS: aroGfbr), fbr-chorismate mutase/prephenate dehydrogenase (fbr-CM/PDH: tyrAfbr), phosphoenolpyruvate synthetase (PEPS: ppsA), and transketolase.
This l-tyrosine over-producing strain could produce 4.37 g/L l-tyrosine in a jar fermenter culture. Next, we modified the strain so that the over-produced l-tyrosine was converted to dopamine by tyrosinase (TYR: TYR) and an L-dopa specific decarboxylase (DODC: DDC). These steps differ from the plant pathway. To achieve this conversion, the l-tyrosine over-producing strain was transformed with a plasmid containing TYR and DDC, and the resultant strain produced 1.03 g/L dopamine in a jar fermenter culture. The (S)-reticuline synthetic pathway from dopamine was established in our previous work. Five genes involved in the (S)-reticuline synthetic pathway (monoamine oxidase: MAO, norcoclaurine synthetase: NCS, 6-O-methyltransferase: 6OMT, coeruleine N-methyltransferase: CNMT, and 4-O-methyltransferase: 4OMT) were expressed in the dopamine-producing strain. Thus, the platform strain contained 11 exogenously expressed genes driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter, and one gene knockout. This strain yielded 40 mg/L (S)-reticuline from 15 g glycerol in a jar fermenter culture. The amount of (S)-reticuline produced by this strain was sufficient compared with the amount that could be isolated from plants, but the conversion efficiency of (S)-reticuline from glycerol was quite low (0.15%). Many experiments were required to increase the production rate (e.g., selection of enzyme combinations, optimization of culture conditions, and alteration of host genotypes). Although we have developed a (S)-reticuline-producing system in a jar fermenter culture, it is difficult to manipulate this system with numerous samples in parallel. In contrast, bench-top cultures of the (S)-reticuline-producing strain are easier to manipulate with multiple samples. We therefore used this strategy to improve the production system.

**Bench-Top Production of (S)-Reticuline**

In our previous study, the (S)-reticuline-producing strain was cultured in Turbo Broth (Athena Enzyme System). Among all the media provided in the Media Optimization Kit (Athena Enzyme System), this was the most suitable medium for dopamine production from the dopamine-producing strain (BL21DE3AthyR containing an l-tyrosine over-producing pathway, tyrosinase, and dopa decarboxylase). However, we had not screened different media in small-scale cultures for (S)-reticuline production. For bench-top production of (S)-reticuline, we first screened several types of conventional media; LB medium, 2 × YT medium, Terrific Broth (Sigma–Aldrich), and Turbo Broth. All media contained 30 μM CuSO₄, 20 g/L glycerol, and appropriate antibiotics. LB and 2 × YT media also contained 2.2 g/L monopotassium dihydrogen phosphate and 9.4 g/L dipotassium hydrogen phosphate for buffering as with Terrific Broth and Turbo Broth. An overnight culture of the (S)-reticuline-producing strain was diluted 100-fold into 50 mL of each medium in a 300-mL baffled shake flask, and the cultures grown at 25°C with shaking at 150 rpm. We added IPTG (0.1 mM) at 12 h after inoculation (early stationary growth phase). The concentration of (S)-reticuline was measured as described previously. The Terrific Broth-based medium was the most suitable for (S)-reticuline production, yielding 15.1 mg/L (Fig. 2A). The yield was quite low from the Turbo Broth-based medium, even though this medium gave high yields of (S)-reticuline in the jar fermenter system. In fact, the manufacturer had changed the composition of Turbo Broth since we conducted our previous work, and the amount of (S)-reticuline produced using the new formulation of Turbo Broth was insufficient even in the jar fermenter system (data not shown; communication with Athena Enzyme System).

Next, we tried to optimize the IPTG concentration for (S)-reticuline production. Previously, we observed that induction at the logarithmic growth phase resulted in arrest of bacterial growth and a reduction in the amount of (S)-reticuline produced (data not shown); therefore, IPTG was added at the early stationary phase (OD₆₀₀ = 8.0; 12 h after inoculation). To determine the optimum concentration of IPTG, we measured the amount of (S)-reticuline produced after addition of 0, 10, 30, 100, or 300 μM IPTG. For these experiments, we usedTerrific Broth-based medium. (S)-reticuline was produced even with no addition of IPTG, as was observed previously in the jar fermenter culture (Fig. 2B). The maximum production of (S)-reticuline (19.7 mg/L) was induced by addition of 10 μM IPTG. Concentrations of IPTG > 10 μM slightly decreased (S)-reticuline production. Thus, the optimum concentration of IPTG was 10 μM, and this concentration was used in further experiments. A total of 10 μM of IPTG is a relatively low concentration. Hence, we expected that this concentration would not cause growth inhibition even after addition at the logarithmic growth phase. Therefore, we investigated the optimum starting time of IPTG induction. When IPTG was added at the early log phase (OD₆₀₀ = 0.6) or mid-log phase (OD₆₀₀ = 2.0), growth arrest was not observed (data not shown); however, the amounts of (S)-reticuline production were relatively low compared with the addition at early stationary phase (OD₆₀₀ = 8.0) or stationary phase (approx. OD₆₀₀ = 30) (Fig. 2C). Because (S)-reticuline production was almost identical between induction at early stationary and stationary phases, we judged that IPTG induction should be done at the time between early stationary and stationary phases.

The (S)-reticuline production pathway of the platform strain includes two reactions that require molecular oxygen: conversion of l-tyrosine to L-dopa, and conversion of dopamine to 3,4-dihydroxyphenylacetaldehyde (Fig. 1). Oxygen is required for these steps, so the amount of aeration was expected to affect (S)-reticuline production. In addition, air supply is one of the biggest differences between small-scale and jar-fermenter cultures. To optimize aeration, the (S)-reticuline-producing strain was cultured at 100, 150, 200 or 250 rpm. Terrific Broth-based medium was used for these experiments, and 10 μM IPTG was used for induction when the OD₆₀₀ reached 8.0. We anticipated that the highest rotation speed would be most effective.
Figure 1. Bacterial BIA biosynthetic pathway constructed in *Escherichia coli*. A part of a simple carbon source is catabolized to PEP and E4P via glycolysis and the pentose phosphate pathway, respectively. These two compounds are converted to \( \text{L-tyrosine} \) in the shikimic acid pathway. \( \text{L-tyrosine} \) is converted to \((S)-\text{reticuline}\) via seven steps. Abbreviations: E4P, erythrose-4-phosphate; HPP, 4-hydroxyphenylpyruvate; 3,4-DHPAA, 3,4-dihydroxyphenylacetalddehyde; fbr-DAHPS, feedback-inhibition-resistant (fbr) 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (aroG\(^{fbr}\), GenBank\(^{TM}\) accession number J01591); fbr-CM/PDH, fbr-chorismate mutase/prephenate dehydrogenase (tyrA\(^{fbr}\), GenBank accession number M10431); PEPS, phosphoenolpyruvate (PEP) synthetase (ppsA, GenBank accession number X59381); TKT, transketolase (tktA, GenBank accession number X68025); TYR, tyrosinase of *Ralstonia solanacearum* (Rcs0337, GenBank accession number AL646052); DODC, DOPA decarboxylase of *Pseudomonas putida* (GenBank accession number AE015451); MAO, monoamine oxidase of *Micrococcus luteus* (GenBank accession number AB010716); NCS, norcoclaurine synthetase of *Coptis japonica* (GenBank accession number AB267399); 6OMT, norcoclaurine 6-O-methyltransferase of *C. japonica* (GenBank accession number D29811); CNMT, coclaurine N-methyltransferase of *C. japonica* (GenBank accession number AB061863); and 4'OMT, 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase of *C. japonica* (GenBank accession number D29812).
for (S)-reticuline production, but found that 150 rpm was the optimum speed (Fig. 3). (S)-reticuline production was negatively affected by rotation speeds > 150 rpm. Excessive bubbling occurred in the cultures at 200 rpm and 250 rpm; therefore, cells might be damaged in the cultures at higher rotation speeds, resulting in inhibition of (S)-reticuline production.

We found that glycerol was exhausted before 36 h after inoculation, and (S)-reticuline accumulation ceased simultaneously (Fig. 3B). Therefore, we anticipated that feeding glycerol might increase the yield of (S)-reticuline. We added 1 g glycerol (final concentration: 20 g/L) to the culture (with shaking at 150 rpm) at 36 h after inoculation. As expected, we observed continuous accumulation of (S)-reticuline after feeding, and obtained a maximum yield of 33.9 mg/L, ~1.5-fold greater than that in the culture that was not fed with glycerol (Fig. 3B, compare diamonds at 60 h with triangles at 36 h). The cell mass in the glycerol-fed culture was 1.3-fold greater than that of the non-fed culture (Fig. 3A), suggesting that increased production was due to increased cell mass. Further feeding of glycerol was not efficient for (S)-reticuline production and cell mass, probably due to decreased glycerol consumption after 60 h (data not shown). Using these experiments, we achieved bench-top (S)-reticuline production with a yield of 33.9 mg/L in 60 h, a similar yield to that obtained using the jar fermenter culture (Fig. 3B). In these small-scale cultures and in the jar fermenter culture, we did not detect significant amounts of intermediate compounds (L-dopa, dopamine, norlaudanosoline, mono- and dimethylated norlaudanosoline) in the culture medium.

The optimum culture conditions for bench-top production of (S)-reticuline can be summarized as follows: an overnight culture is diluted 100-fold into 50 mL Terrific Broth containing 30 mM CuSO₄, 20 g/L glycerol, 50 mg/L ampicillin, 25 mg/L kanamycin, and 50 mg/L chloramphenicol in a 300-mL baffled shake flask. Cells are grown at 25°C with shaking at 150 rpm. IPTG is added to a final concentration of 10 μM to induce (S)-reticuline production after the OD₆₀₀ reaches 8.0, and 20 g/L glycerol is added 36 h after inoculation. Using this bench-top method, a sufficient amount of (S)-reticuline can be readily prepared by research teams that do not have a jar fermenter system. In addition, this system enables experiments with numerous samples (e.g., experiments assessing the effects of mutagenesis, types/ratios of enzymes) to be conducted in parallel. Thus, the bench-top method for production of (S)-reticuline may facilitate research on BIAs.

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Figure 3. Optimization of rotation speed for (S)-reticuline production and effect of glycerol feeding. (S)-Reticuline-producing strain was cultured with shaking at 100 rpm (squares), 150 rpm (triangles and diamonds), 200 rpm (crosses), and 250 rpm (circles). Feeding of glycerol (20 g/L) was conducted 36 h after inoculation (diamonds). Cell growth (A) and (S)-reticuline production (B) are shown. Cells were grown in Terrific Broth-based medium at 25°C and 10 μM IPTG was used for induction when OD<sub>600</sub> reached 8.0. Error bars indicate the standard deviation of three independent experiments.