Higher plants produce diverse chemicals, including alkaloids, terpenoids, and phenolic compounds (phenylpropanoids and flavonoids) as secondary metabolites. These chemicals are widely used for human health and nutrition. Alkaloids, for example, are valued in medicine due to their high biological activities, but most of these metabolites accumulate at low levels in plant cells, resulting in poor extraction yields. Increasingly, attention is devoted to the production of plant metabolites by reconstructing plant biosynthetic pathways in microorganisms. This technology has been aided by advances in synthetic biology and metabolic engineering. Here, the review a fermentation platform for low-cost production of numerous alkaloids using bioengineered Escherichia coli and/or Saccharomyces cerevisiae.

Key words: benzylisoquinoline alkaloid; reticuline; fermentative production; synthetic biology; metabolic engineering

Among these chemicals, alkaloids are highly valued in medicine due to their biological activities. Alkaloids are low-molecular-weight, nitrogen-containing compounds found in about 20% of plant species. Most are derived from amines produced by decarboxylation of amino acids, including histidine, lysine, ornithine, tryptophan, and tyrosine. One of the largest and most diverse groups of pharmaceutical alkaloids is the benzylisoquinoline alkaloids (BIAs). These include the analgesic compounds morphine and codeine and the antibacterial agents berberine and palmatine, which are produced via (S)-reticuline from L-tyrosine in Papaveraceae, Berberidaceae, Ranunculaceae, Magnoliaceae, and many other plant families. (S)-Reticuline is a major intermediate branch point in BIA biosynthesis. In addition, it acts as a non-narcotic building block in the development of novel antimalarial and anticancer drugs.

Plant secondary metabolites (including BIAs) are usually obtained by extraction, but yields are poor because the metabolites accumulate at low levels in plant cells. Plant metabolic engineering is a popular approach by which to increase alkaloid yields, but the effort to obtain the desired products is thwarted by complicated and strict regulation of metabolic flows. An alternative means of producing secondary metabolites (including BIAs) is chemical synthesis, but this approach is hampered by the complexity and chirality of the compounds, which precludes the development of cost-effective methods. Recently, the reconstruction of plant biosynthetic pathways in microorganisms has received increasing attention. Microbial systems can improve not only the quantity but also the quality of the products, because they do not accumulate undesired metabolites when operating correctly.

Among plant secondary metabolites, isoprenoids are produced from acetyl-CoA in the mevalonate pathway, or from glyceraldehyde-3-phosphate and pyruvic acid in the 1-deoxy-d-xylulose-5-phosphate (DXP) pathway (Fig. 1). The biosynthesis of isoprenoids is well characterized, and these compounds are produced by microbes in a simple growth medium. The other major groups of secondary metabolites, alkaloids and...
phenolic compounds, are derived from aromatic amino acids (Fig. 1). Among these, various phenolic compounds have been produced successfully from L-phenylalanine and L-tyrosine in microbial systems.14 In this review, the focus is on current knowledge of the metabolic engineering and fermentative production of BIAs from simple carbon sources.

I. Metabolic Engineering of BIAs in Microbes

Various BIAs are produced from L-tyrosine via (S)-reticuline in plants. The biosynthetic pathway of (S)-reticuline from L-tyrosine is shown in Fig. 2. Firstly, L-tyrosine is converted to dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA). These products combine to form (S)-norcoclaurine (1a), assisted by norcoclaurine synthase (NCS, EC 4.2.1.78).15–21 The basic skeleton of the BIA molecule, (S)-norcoclaurine (1a), is converted into (S)-reticuline in four reaction steps; (i) 6-O-methylation of norcoclaurine (1a) to coclaurine (2a) by norcoclaurine 6-O-methyltransferase (6OMT, EC 2.1.1.128), (ii) N-methylation of coclaurine (2a) by coclaurine N-methyltransferase (CNMT, EC 2.1.1.140), (iii) hydroxylation of the 3'-C in N-methylcoclaurine (3a) by CYP80B, and (iv) 4'-O-methylation of 3'-hydroxy-N-methylcoclaurine (3b) by 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'OMT, EC 2.1.1.116). The biosynthetic pathway from L-tyrosine to (S)-norcoclaurine (1a) is branched, and coupling of dopamine and 4-HPAA can occur relatively readily by a chemical reaction, but efficiently reconstructing the biosynthetic pathway from L-tyrosine to (S)-reticuline remains a significant challenge. Furthermore, it is often difficult to produce active forms of plant cytochrome P450 enzymes (such as CYP80B) in bacteria. Hence researchers seek alternative ways of biosynthesizing these compounds, such as adopting enzymes from other organisms or designing a shortcut pathway. To this end, we designed a tailor-made pathway for BIA production in microbes (Fig. 3). It uses five reaction steps for the biosynthesis of (S)-reticuline via norlaudanosoline (1b) with dopamine as substrate.

At first step, dopamine is de-aminated to 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA). This reaction is catalyzed by a monoamine oxidase (MAO) enzyme. Since eukaryotic MAO is a membrane protein, it is not easily expressed in E. coli. Hence we use microbial Micrococcus luteus MAO, which is soluble in the cytosol.22 Next, dopamine and 3,4-DHPAA are combined into norlaudanosoline (1b) via the Pictet–Spengler reaction, catalyzed by norcoclaurine synthase (NCS). NCS catalyzes isouquinoline formation from the condensation reaction between aldehyde carbonyls (such as 4-HPAA) and dopamine. We have isolated and characterized two types of NCS (CjNCS1 and CjPR10A) from Coptis japonica cells.23 The activity of the recombinant CjNCS1 enzyme expressed in E. coli cells is considerably lower than that of the native enzyme in plant cells. Conversely, an active form of CjPR10A was sufficiently expressed in E. coli. Hence in designing the microbial production system, we selected CjPR10A as the NCS enzyme. Finally, norlaudanosoline (1b) is successively methylated to 6-O-methylnorlaudanosoline (3-hydroxycoclaurine) (2b), 6-O-methyllaudanosoline (3'-hydroxy-N-methylcoclaurine) (3b), and reticuline by three different methyltransferases (6OMT, CNMT, and 4'OMT, respectively). In the modified biosynthetic pathway, reticuline is produced by means of selected enzymes.23

E. coli cells containing the reticuline biosynthetic genes in the artificial pathway were constructed with two types of plasmid vector. The MAO and NCS genes were inserted into pKK223-3, while those of 6OMT, CNMT, and 4'OMT were inserted into pACYC184. The E. coli strain was cultured in a medium supplemented with 5 mM dopamine, and it yielded 11 mg/L of (R,S)-reticuline within 28 h23 (Table 1). Although the reticuline produced in this strain is a racemic mixture, NCS produces only the (S)-form in a stereospecific manner, suggesting that NCS cannot function efficiently in this strain, and that norlaudanosoline (1b) forms by a spontaneous condensation reaction. Subsequent transformations (a series of methylations) in both enantiomers occurred at nearly equal efficiency to give a racemic reticuline. In support of this conjecture, E. coli cells expressing the reticuline biosynthetic gene without NCS produced racemic reticuline at the same level. The overall yield of reticuline from dopamine was 2.9%, attributable to the instability of dopamine and certain reaction intermediates that are readily oxidized to a melanin-like pigment. An advantage of fermentative (R,S)-reticuline is that no additional SAM (a methyl donor for methyltransferases) is required, because microbial cells regenerate SAM naturally to maintain in vivo methylation activity during bioconversion.24
For (S)-reticuline production, crude enzymes from transgenic E. coli cells were prepared and treated with dopamine and SAM. In this reaction, stereospecific (S)-reticuline was synthesized from dopamine via crude enzymes, requiring no fine tuning of enzyme levels or enzyme purification. The in vitro biomanufacturing system produced 55 mg/L (overall yield 14.4%) of (S)-reticuline from 5 mM dopamine within 1 h \(^{23}\) (Table 1). Hence this system can produce optically active (S)-reticuline within a much shorter time (1 h) than methods using cultured plant cells or transgenic plants (which can require months to years).

(S)-Reticuline is the intermediate of a range of BIAs. To produce BIAs from (S)-reticuline, we constructed a BIA biosynthetic pathway using genes of the berberine bridge enzyme (BBE) or the diphenyl ring bridging enzyme (corytuberine synthase: CYP80G2) plus CNMT (which has relatively broad substrate specificity and N-methylates corytuberine to magnoflorine) to produce two BIAs: magnoflorine and scoulerine (Fig. 3). However, the transformation of reticuline to different alkaloids often requires P450 enzymes, which are difficult to express in active form in E. coli. This problem was averted by two-step synthesis of alkaloids in E. coli and S. cerevisiae cells.\(^{23}\) For magnoflorine production, transgenic E. coli cells expressing reticuline biosynthetic genes were cultured in a medium supplemented with 5 mM dopamine. S. cerevisiae cells exp-
expressing CYP80G2 and CNMT were added to the *E. coli* culture medium after a specified period. The magnoflorine yield was 7.2 mg/L of culture (overall yield 1.9%) within 72 h. They also added *Arthrobacter globiformis*, *Brevibacterium lactofermentum*, and *Corynebacterium glutamicum* have been engineered by traditional mutagenesis. 

II. Fermentative Production of BIAs from Simple Carbon Sources

**Production of a wide array of BIA metabolites.** They used simple carbon sources, conversion of L-tyrosine to dopamine, and BIA overproduction in *E. coli* cells. These results suggest that the proposed combination system benefits the synthesis of various BIAs.

Following the presentation of our research, Hawkins and Smolke also engineered yeast strains expressing combinations of enzymes from three plant sources (*Thalictrum flavum*, *Papaver somniferum*, and *Arabidopsis thaliana*) and humans as microbial hosts for the production of a wide array of BIA metabolites. They examined the ability of different combinations of three recombinant enzymes from *T. flavum* and *P. somniferum* to produce (R,S)-reticuline from (R,S)-norlaudanosoline (1b). They reported that (R,S)-norlaudanosoline (1b) was converted to (R,S)-reticuline at a yield of 164.5 mg/L of culture by inserting methylintransferase (6OMT, CNMT, 4OMT) genes from *T. flavum* and *P. somniferum* into *S. cerevisiae*. In addition, they described a new enzyme-tuning strategy that can be applied generally to determine optimal enzyme expression levels to conserve cellular resources and to improve growth and production rates without compromising pathway flux. Three additional enzymes from *T. flavum* and *P. somniferum* and a reductase partner from *A. thaliana* were also expressed in *S. cerevisiae*, with production of (S)-scoulerine (65.4 mg/L), (S)-tetrabutyldolcumbamine (68.2 mg/L), and (S)-tetraphydroberberine (33.9 mg/L) respectively from (S)-reticuline. Furthermore, an intermediate of morphine biosynthesis, salutaridine, was produced from (R,S)-reticuline at a yield of 24.5 mg/L of culture with a human P450 enzyme (CYP2D6), demonstrating a novel activity for this P450 enzyme.

II. Fermentative Production of BIAs from Simple Carbon Sources

While BIA production in microorganisms has been rendered possible by advances in synthetic biology and metabolic engineering, the production cost of BIA production from dopamine and from norlaudanosoline (1b) remains high because these substrates are relatively expensive and easily oxidized, forming insoluble brown polymers during cultivation. Consequently, such methodologies are commercially unfeasible. The conversion of less expensive material (such as glucose and glycerol) into BIAs is an important challenge of secondary metabolite engineering. To address it, we developed a strategy for BIA fermentation from simple carbon sources in *E. coli* cells. BIA fermentation occurs in three steps: 1-tyrosine fermentation from simple carbon sources, conversion of 1-tyrosine to dopamine, and BIA production from dopamine via (S)-reticuline.

1-tyrosine is used to synthesize various compounds, including certain drugs, melanins, biodegradable resins, and phenylpropanoids. Hence various overproducing strains of 1-tyrosine, including *Arthrobacter globiformis*, *Brevibacterium lactofermentum*, and *Corynebacterium glutamicum* have been engineered using different strategies. 

*E. coli* is one of the most thoroughly studied organisms, and its genome can be manipulated by a range of molecular genetic methods, enabling the construction of desired mutants for the overproduction of aromatic amino acids. In our research, 1-tyrosine overproduction was accomplished in three steps of genetic engineering. The *tyrR* gene, whose product represses expression of the genes involved in aromatic amino-acid biosynthesis, has been disrupted in an *E. coli* strain. In addition, the feedback-inhibition-resistant (fbr) 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP) synthase (fbr-DHAPSyn: *aroG*<sup>fr</sup>) and fbr-chloromate mutase/prephenate dehydrogenase (fbr-CM/PDH: *tyrA*<sup>fr</sup>) enzymes have been overexpressed in the shikimic acid pathway. To increase metabolic flow into the shikimic acid pathway, phosphoenolpyruvate synthetase (PEPS: *ppsA*) and transketolase (TKT: *tktA*<sup>fr</sup>) can be introduced exogenously (Fig. 4). Thus the 1-tyrosine over-producing *E. coli* strain contains a knockout *tyrR* mutation and a plasmid expressing the *aroG*<sup>fr</sup>, *tyrA*<sup>fr</sup>, *ppsA*, and *tktA* genes. With glycerol as sole carbon source, engineered *E. coli* cells produced about 4.37 ± 0.17 g/L of 1-tyrosine in the medium.

The second step of BIA fermentation is the conversion of 1-tyrosine to dopamine. In plants, 1-tyrosine is converted mainly to L-DOPA by tyrosine hydroxylase (TH), followed by the conversion of L-DOPA to dopamine by tyrosine/DOPA decarboxylase. However, TH requires a co-factor, tetrahydrobiopterin, which is not synthesized in *E. coli* cells. Furthermore, tyrosine/DOPA decarboxylase catalyzes the decarboxylation of 1-tyrosine and L-DOPA to tyramine and dopamine respectively. Tyramine is unwanted in microbial BIA synthetic pathways because its MAO product (4-HPAA) combines with dopamine to form norcoclaurine (1a), which requires CYPS80B for reticuline conversion. The BIA synthetic pathway from 1-tyrosine to dopamine is hence difficult to construct using TH and tyrosine/DOPA decarboxylase. Originally, we designed an artificial pathway using microbial enzymes. To convert 1-tyrosine to 1-DOPA in the microbial system, we selected tyrosinase (TYR, EC 1.14.18.1) from *Ralstonia solanacearum*, which is unrelated to BIA biosynthesis. To convert 1-DOPA to dopamine, we selected the 1-DOPA-specific decarboxylase (DODC; EC 4.1.1.28) from *Pseudomonas putida* strain KT2440, which exhibited a more than 10<sup>3</sup>-fold preference for 1-DOPA over other aromatic amino acids. When the selected TYR and DODC were expressed in the 1-tyrosine overproducing *E. coli* strain, 1.05 g/L of dopamine was produced in the medium.

Reticuline can be produced from simple carbon sources by combining 1-tyrosine fermentation with the artificial BIA synthetic pathway. As the final step in fermentative (S)-reticuline production, we combined the above-mentioned dopamine-producing pathway with a...
previously described synthetic pathway from dopamine to (S)-reticuline.23) The tyrR gene was deleted from the reticuline-producing strain and 11 genes were introduced (aroGfbr, tyrAfbr, ppsA, tktA, TYR, DODC, MAO, NCS, 6OMT, CNMT, and 40OMT) via four plasmid vectors. This strain produces reticuline from glucose and glycerol. Glycerol-fed batch cultures in a jar fermenter system yielded a maximum of 46.0 mg/L (Table 1). The predominant formation of (S)-enantiomer can ascribe to a lack of dopamine, preventing the spontaneous condensation of dopamine and 3,4-DHPAA to (R,S)-norlaudanosoline. This was confirmed by the fact that no dopamine accumulation was detected at any growth phase. In our fermentative system, optically active (S)-reticuline was rapidly produced within a few days, whereas fermentation systems using cultured plant cells or transgenic plants require months to years.

Next we examined bench-top production of (S)-reticuline and optimization of culture conditions in shake flask cultures.40) The bench-top approach is an alternative to the jar fermenter system, which attains comparable yields of (S)-reticuline (33.9 mg/L in 60 h). Thus the bench-top method of (S)-reticuline production can facilitate BIA research. Shake flask cultures make possible the parallel culturing of numerous samples to optimize production conditions.

III. Conclusion

In natural plants, a number of secondary metabolites, especially intermediates of biosynthetic pathways, are present in low quantities. Extraction of secondary metabolites from plant sources is problematic in terms of time required, collection, and scale-up procedures. We consider that our bacterial platform is more suitable for secondary metabolite production than for extracting metabolites from plant sources. The microbial system should open a new research avenue in which microbial cells can be manipulated for the low-cost production of numerous diverse alkaloids. Our proposed fermentation system uses an artificial synthetic pathway in place of the original plant biosynthetic pathway (which remains poorly understood). Furthermore, this modified produc-
tion system enables novel compounds to be synthesized from the pathway. Microbial metabolic engineering has become increasingly important in the industrial-scale production of plant secondary metabolites. Given that many plant secondary metabolites are derived from primary metabolites, such as amino acids or acetyl-CoA, our proposed fermentative system presents a general metabolic engineering strategy for secondary metabolite production, and in particular for aromatic amino-acid derivatives such as alkaloids. As such, our platform represents a significant advance in the metabolic engineering of secondary metabolites.

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