A Novel Process for Obtaining Phenylpropanoic Acid Precursor Using *Escherichia coli* with a Constitutive Expression System

Jing-long Liang¹,², Liqiong Guo¹,², Ping Sun¹,², Binghua Jiang³, Junfang Lin¹,²,⁴, Weixiong Guo¹,², and Hua Wan⁴

¹Department of Bioengineering, College of Food Science and Institute of Food Biotechnology, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou 510640, China
²Joint Research & Development Center for Natural Products of Alchemy Biotechnology Co. Ltd. and South China Agricultural University, Guangzhou 510640, China
³Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA
⁴College of Informatics, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou 510640, China

Abstract Phenylpropanoids are widely used in food supplements, pharmaceuticals, and cosmetics with diverse benefits to human health. *Trans*-cinnamic acid or *p*-coumaric acid is usually used as the starting precursor to produce phenylpropanoids. Synthetic bioengineering of microbial cell factories offers a sustainable and flexible alternative method for obtaining these compounds. In this study, a constitutive expression system consisting of *Rhodotorula glutinis* phenylalanine/tyrosine ammonia lyase was developed to produce a phenylpropanoic acid precursor in *Escherichia coli*. To improve *trans*-cinnamic acid and *p*-coumaric acid production, BioBrick optimization was investigated, causing a 7.2- and 14.2-fold increase in the yield of these compounds, respectively. The optimum strain was capable of *de novo* producing 78.81 mg/L of *trans*-cinnamic acid and 34.67 mg/L of *p*-coumaric acid in a shake flask culture. The work presented here paves the way for the development of a sustainable and economical process for microbial production of a phenylpropanoic acid precursor.

Keywords: phenylpropanoic acid precursor, phenylpropanoids, *escherichia coli*, bioprocess design, constitutive expression

Introduction

Phenylpropanoids, which include stilbenoids, flavonoids, curcuminoids, and cinnamyl anthranilate, are a diverse group of secondary metabolites synthesized from *L*-phenylalanine or *L*-tyrosine. These compounds exhibit different bioactivities including antioxidant, antiviral, antibacterial, and anticancer (1-3). Thus, phenylpropanoids are widely used in pharmaceuticals, cosmetics, and food supplements. With the rapid development of market demands, phenylpropanoid supply is facing new opportunities and challenges (4). Phenylpropanoids are traditionally acquired through phytoextraction from mosses or angiosperms (5-7). However, the extraction yield of these compounds can hardly exceed 1% of the dry weight (8). Recently, synthetic biologists have attempted to explore biological phenomena and natural product synthesis by artificial-biological systems (9). Phenylpropanoids biosynthesis in recombinant *Escherichia coli* and *Saccharomyces cerevisiae* has achieved great progress, especially the biosynthesis of several bioactive compounds such as caffeic acid (10), naringenin (11,12), anthocyanin (13), resveratrol (14), pinocembrin (15,16), pinosylvin (17), and styrene (18) by engineered *E. coli* fermentation. *E. coli*, which is the most genetically tractable microbial host, has been used as a microbial factory for the artificial biosynthesis of different types of molecules (19). The engineering strategy for the biosynthetic pathway of phenylpropanoids in microorganisms is shown in Fig. 1. This strategy involves embedding phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL) to convert endogenous/extrinsic *L*-phenylalanine into *trans*-cinnamic acid or *l*-tyrosine into *p*-coumaric acid. Different pivotal enzymes then convert *trans*-cinnamic acid or *p*-coumaric acid into different phenylpropanoids.

So far, the development of phenylpropanoid biosynthesis in *E. coli* is limited by some problems. Firstly, *trans*-cinnamic acid or *p*-coumaric acid is usually used as the starting precursor to produce phenylpropanoids because of the low turnover numbers of the reaction catalyzed by PAL or TAL, but the cost is more than that incurred using amino acids. Therefore, producing phenylpropanoids from *l*-tyrosine/*l*-phenylalanine or a carbon source has attracted the attention of academic and industrial researchers. Secondly, expression
system inducers such as isopropyl-β-D-thiogalactopyranoside (IPTG) are toxic and unsuitable for the industrial production of phenylpropanoids. By contrast, constitutive expression systems are appropriate for industrial needs. Such systems can be actively induced by the carbon source during fermentation and allow the continuous transcription of heterogeneous genes. For instance, the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (pGAP) system has been successfully used to produce trans-resveratrol in engineered E. coli (14). Thirdly, different strains with different expression vectors have different effects (20,21). BioBrick assembly parts such as different ribosomal binding sites (RBSs), replication regions, promoters, terminators, and polycistronic transcriptional assemblies are also limiting factors that need to be resolved (22).

In this study, the Rhodotorula glutinis Rg PAL/TAL enzyme, which has dual substrate specificity and high enzymatic activity in E. coli (23), was selected for heterogeneous expression. The kinetic analyses were conducted using L-phenylalanine and L-tyrosine as substrates to explore the substrate preference of RgPAL/TAL. The constitutive expression, BioBrick assembly, and strain were optimized to biosynthesize trans-cinnamic acid and p-coumaric acid in E. coli. To the best of our knowledge, this is the first to explore the possibility of using a constitutive expression system for phenylpropanoic acid precursor bioproduction in E. coli. Moreover, this synthetic process may be further used for industrial food bioproduction of phenylpropanoids in E. coli.

Materials and Methods

Reagents, chemicals, and strains E. coli DH5α was used for plasmid cloning. E. coli ATCC31884 and other strains (Table 1) were used for recombinant molecule production. R. glutinis GIM2.156 was used for total RNA isolation. PrimeScript reverse transcription polymerase chain reaction (RT-PCR) kit, ExTaq polymerase, vector plasmid pMD18-T, restriction enzymes, and T4 DNA ligase were purchased from Takara Biochemicals Inc, Dalian, China. Vector plasmids pQE-30 and pREP4 were purchased from Qiagen Inc, Shanghai, China. DNA plasmids were prepared from stock strains using a Tiangen plasmid miniprep kit, Beijing, China. DNA fragments were isolated through gel extraction using a Tiangen gel DNA recovery kit (Tiangen Biotech Co., Ltd., Beijing, China). L-tyrosine, L-phenylalanine, trans-cinnamic acid, and p-coumaric acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

RgPAL/TAL complementary DNA (cDNA) cloning Total RNA was extracted in accordance with the hot phenol method. The first-strand cDNA of RgPAL/TAL was synthesized by using the PrimeScript RT-PCR kit in accordance with the manufacturer’s protocol. The pF_PAL/TAL_clone and pR_PAL/TAL_clone specific primers listed in Table 1, based on existing PAL sequences (Genebank entry KF770992), were used to amplify the complementary strand. The RT-PCR products were purified and ligated into the pMD18-T vector to generate pMD18-RgPAL/TAL for sequencing.

Expression plasmid construction Plasmid pMD18-pGAP was provided by Bioengineering Laboratory stocks. The gap promoter (pGAP) was cloned from pMD18-pGAP. The mmb T1 terminator was cloned from pQE-30. RgPAL/TAL was cloned from pMD18-RgPAL/TAL. The total RgPAL/TAL operon (fusion of three DNA fragments) was amplified through touchdown overlap extension PCR. Different sequences of RBS were added using specific primers. Different RgPAL/TAL operon fragments were digested by HindIII and XhoI and independently inserted to the HindIII/XhoI site of pQE-30 or pREP4.