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Biosynthesis of resveratrol using metabolically engineered *Escherichia coli*

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Abstract

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a phenolic compound widely used in pharmaceutics and nutraceutics. Although resveratrol is produced by some plant species, including grapes, peanuts, and berries, the content of resveratrol and its derivatives are very low. Therefore, an alternative biosynthetic method using microorganisms, such as *Escherichia coli*, has been developed over the past two decades. In the present study, a resveratrol-over-producing *E. coli* strain was developed using three strategies. First, we increased the synthesis of *p*-coumaric acid, a precursor of resveratrol, by manipulating genes in the shikimate pathway of *E. coli*. Second, three genes involved in resveratrol biosynthesis, such as tyrosine ammonia lyase (*TAL*), 4-coumaroyl CoA ligase (*4CL*), and stilbene synthase (*STS*), were cloned from diverse sources, such as plants and microorganisms, and the best combination was selected to maximize resveratrol production in *E. coli*. Finally, culture conditions, such as cell concentration, culture temperature, and carbon sources, were established for optimal resveratrol production. Through these strategies, approximately 80.4 mg/L of resveratrol was biosynthesized after 48 h of culture using glycerol as a carbon source.

Keywords: *Escherichia coli*, Metabolic engineering, Phenolic compounds, Resveratrol

Introduction

Polyphenols are plant secondary metabolites and valuable sources for the development of cosmetic materials, functional food ingredients, and pharmaceuticals [1–3]. Polyphenols are generally derived through the phenylpropanoid pathway [4]. Depending on their carbon skeleton, they can be classified into four classes, such as phenolic acids including gallic acid and salicylic acid composed of C_6-C_1 skeleton, hydroxycinnamic acid including cinnamic acid, *p*-coumaric acid, and caffeic acid composed of C_6-C_3 skeleton, stilbene including resveratrol, piceatannol, and pallidol composed of C_6-C_2-C_6 skeleton, flavonoids including naringenin, quercetin, and genistein composed of C_6-C_3-C_6 skeleton [4–6].

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenolic compound containing a C_6-C_2-C_6 skeleton. It is synthesized naturally in several plants in response to pathogenic fungi and bacteria or wounds caused by insects and herbivores [7–9]. Resveratrol was first isolated from the white hellebore roots and subsequently isolated from *Polygonum cuspidatum* [10, 11]. Most of the commercial resveratrol throughout the world is extracted from *P. cuspidatum* E. et Z. In recent years, the biological effects of resveratrol on atherosclerosis and coronary heart disease, and its anticancer, antileukemic, and immune-modulating activities have been well documented through a variety of physiological and pharmacological studies [12, 13]. In plants, resveratrol is synthesized from the aromatic amino acid phenylalanine through the sequential reaction of four enzymes, phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumaroyl-CoA synthase (4CL), and stilbene synthase (STS) (Fig. 1) [14]. PAL is responsible for the deamination of L-phenylalanine to produce trans-cinnamic acid. Subsequently, trans-cinnamic acid is converted into *p*-coumaric acid (4-hydroxy cinnamic

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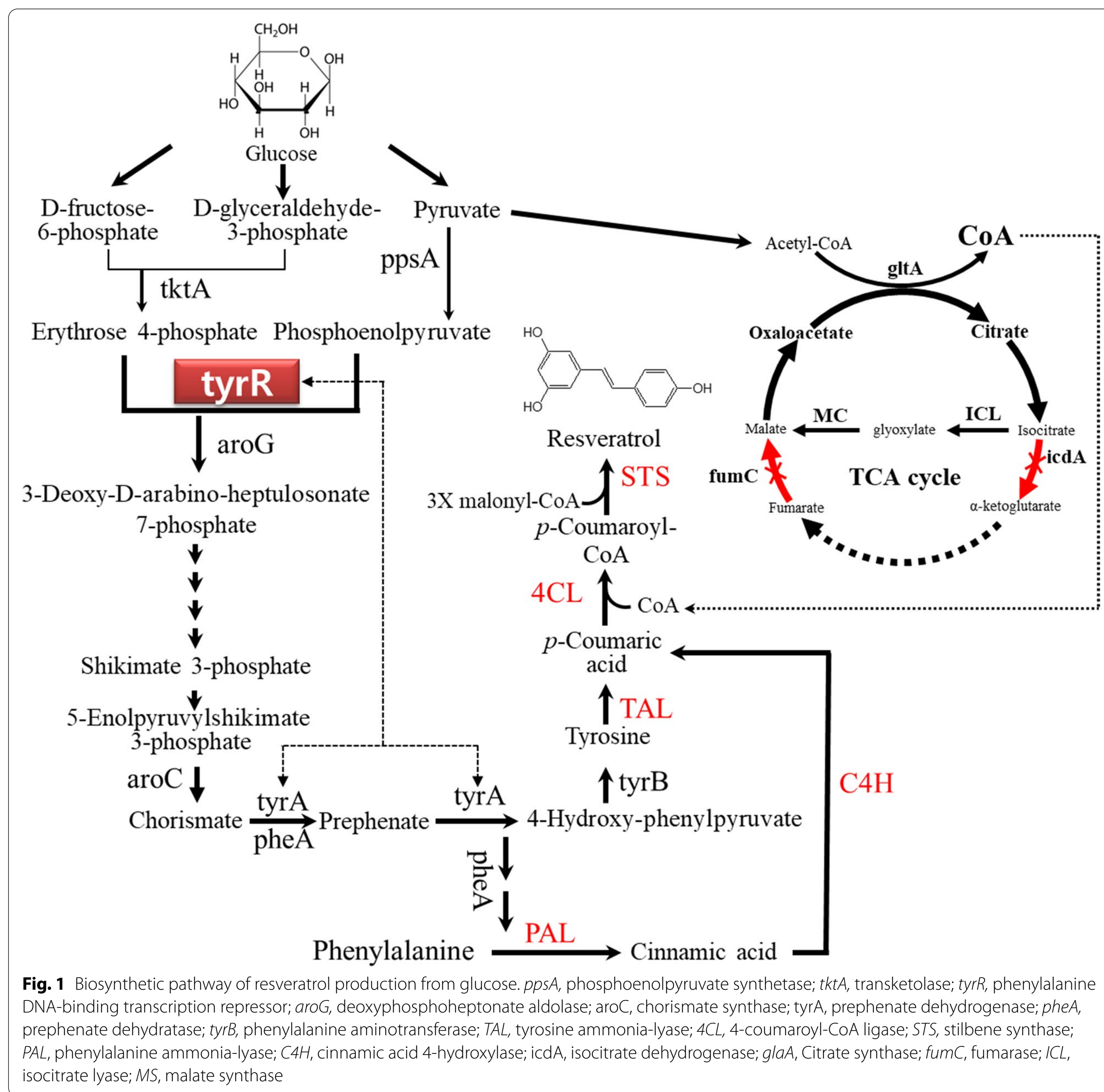

Fig. 1 Biosynthetic pathway of resveratrol production from glucose. *ppsA,* phosphoenolpyruvate synthetase; *tktA,* transketolase; *tyrR,* phenylalanine DNA-binding transcription repressor; *aroG,* deoxyphosphoheptonate aldolase; aroC, chorismate synthase; tyrA, prephenate dehydrogenase; *pheA,* prephenate dehydratase; *tyrB,* phenylalanine aminotransferase; *TAL,* tyrosine ammonia-lyase; *4CL,* 4-coumaroyl-CoA ligase; *STS,* stilbene synthase; *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamic acid 4-hydroxylase; icdA, isocitrate dehydrogenase; *glaA*, Citrate synthase; *fumC*, fumarase; *ICL*, isocitrate lyase; *MS*, malate synthase

acid) by C4H (Fig. 1). *p*-Coumaric acid is converted into *p*-coumaroyl-CoA by 4CL with coenzyme A as a co-substrate. *p*-Coumaroyl-CoA is condensed with three malonyl-CoA units through the sequential reaction of STS, belonging to polyketide III (PKS III), to produce resveratrol [15]. Because the functional expression of C4H (Fig. 1), a member of the P450 genes, is generally difficult in *E. coli,* tyrosine ammonia lyase (TAL), which catalyzes the deamination of tyrosine to *p*-coumaric acid, has been recently employed for the biosynthesis of various polyphenols [16]. To synthesize resveratrol from tyrosine in *E. coli*, three genes, *TAL*, *4CL*, and *STS*, are required (Fig. 1). However, tyrosine is a limiting factor for the synthesis of resveratrol from glucose. To increase the tyrosine content in *E. coli*, metabolic engineering approaches have been applied in *E. coli*. [17, 18].

Over many decades, natural compounds derived from plants have been the materials of great medicinal value associated with human health benefits [19, 20]. However, there are some obstacles that must be overcome, including seasonal and spatial limitations, low yields, and complicated refining processes, such as extraction

and isolation, before the commercial usage of these biologically active molecules [21]. For these reasons, some alternative production methods have been developed, including plant cell or hairy root culture and simple biotransformation or de novo synthesis using microorganisms, such as *E. coli* and yeast, for the production of pharmaceutically useful substances of plant origin [18, 22–24]. Among them, *E. coli* is an excellent host for producing large-scale commercial commodities because their metabolic pathway is well known and various techniques of gene manipulation are well established [26]. In addition, the synthesized phenolic compounds can be easily purified using simple organic solvents such as ethyl acetate [18, 25]. Using *E. coli,* a number of medicinally important compounds, such as hydroxycinnamic acids, coumarins, stilbenoids, flavonoids, terpenoids, and alkaloids, have been successfully synthesized [18, 27–29, 35].

Most of the previous studies related to the production of resveratrol in *E. coli* started from *p*-coumaric acid. The titer was approximately 16 mg/L resveratrol from 800 mg of *p*-coumaric acid, when *E. coli* BL21 (DE3) containing the gene *4CL* from *Nicotiana tabacum* and the gene *STS* from *Vitis vinifera* was used [30], whereas when the *E. coli* JM109 strain transformed with the gene *4CL* from *Arabidopsis thaliana* and *STS* from *Arachis hypogaea* was used, the amount of resveratrol was over 100 mg/L [31]. Lim et al. [32] developed a metabolically engineered *E. coli* strain capable of producing high-yield resveratrol (2.3 g/L) by feeding on 15 mM *p*-coumaric acid. However, because *p*-coumaric acid is relatively expensive compared to glucose, the synthesis of resveratrol from glucose, a renewable source, is an attractive alternative approach. Recently, resveratrol was successfully synthesized from glucose by introducing three genes, *TAL*, *4CL*, and *STS*, into *E. coli* [28]. Yuan et al. [33] reported resveratrol production from glucose using modular engineering of an *E. coli-Saccharomyces cerevisiae* co-culture, and the yield of resveratrol was 28.5 mg/L. Wang et al. [28] reported the production of 114.4 mg/L of resveratrol from glucose, but 3 mM tyrosine was added to the to the culture medium for resveratrol biosynthesis. Although previous studies have demonstrated the de novo production of resveratrol from glucose, the potential to improve productivity remains.

In this work, we describe microbial resveratrol production from glucose using *E. coli*. We engineered the tyrosine biosynthesis pathway for the stable supply of tyrosine by metabolic engineering, introduced three resveratrol biosynthetic genes, and selected the optimal combination of resveratrol biosynthetic genes. In addition, in previous studies, a mutant was used to supply more precursor (tyrosine) for resveratrol biosynthesis [16, 28], but in this study, we used not only *E. coli* mutant for tyrosine but also overexpressed genes for tyrosine biosynthesis in order to synthesize resveratrol from glucose (without supplying tyrosine). Then, we optimized the fermentation conditions such as carbon source, initial cell density, and culture temperature. Finally, the combined effect of genetic modification and culture conditions increased the final resveratrol titer to 80 mg/L. The strategy described here will be applicable as an alternative production tool of resveratrol, which can be used in various applications in food, pharmaceutical, and nutraceutical industry.

Materials and methods

Plasmid constructs

The TAL gene from *Saccharothrix espanaensis* (*SeTAL*) was previously cloned between *Eco*RI and *Not*I sites of pCACYCDuet vector [18]. Three genes of *4LC* from *Streptomyces coelicolar* A(3) (*Sc4CL*), *Oryza sativa* (*Os4CL*), and *Lithospermum erythrorhizon* (*Le4C*L) were cloned between *Bam*HI and *Not*I of pCDFDuet, and named PC-Sc4CL, PC-Os4CL, and PC-Le4CL. The gene *4CL* from *Petroselinum crispum* (*Pc4CL*) was cloned between *the Eco*RI and *Not*I sites of pCDFDuet and named PC-Pc4CL. *STS* from *Vitis vinifera* (VvSTS) was introduced into multiple cloning site 2 using *Eco*RV and *Xho*I sites of PC-Sc4CL, PC-Os4CL, PC-Pc4CL, and PC-Le4CL. The resulting plasmids, in which each gene was controlled by the T7 promoter, were named PC-Sc4CL-VvSTS(P), PC-Os4CL-VvSTS(P), and PC-Le4CL-VvSTS(P) (Table 1). Two STS genes from *Arachis hypogea* (*AhSTS*) and *Picea abies* (*PaSTS*) were cloned into *Eco*RV and *Xho*I of PC-Os4CL, respectively, and named PC-Os4CL-AhSTS(P) and PC-Os4CL-PaSTS(P), which are controlled by an independent T7 promoter (Table 1). In the case of *AhSTS*, since it has a *Hin*dIII restriction enzyme site at the 855 position, a silencing mutation was performed using PCR. To generate a construct in which two genes were controlled by one promoter, *AhSTS*, *VvSTS*, and *PaSTS* were amplified using each primer set containing *Eco*RI and *Hin*dIII sites and cloned into the corresponding sites of the pCDFDuet vector. *Os4CL* was amplified with a forward primer including a ribosomal binding site (RBS) and a *Not*I site such as 5-ATAAGCTTaggaggattacaaaATGATCACGGTGGCG-3 (the underlined part indicates the *Hin*dIII site and the RBS is shown in lower case), and the reverse primer including a *Not*I site such as 5-ATGCGGCCGCTTAGCTGCTTTTGGGCGCATC-3 (the underlined part indicates the *Not*I site) (Table 1). The resulting PCR product was digested with *Hin*dIII I and *Not*I of the restriction enzyme after gel purification and the corresponding sites of PCDFDuet vector containing *AhSTS*, *VvSTS*, or *PaSTS*. The resulting constructs, PC-AhSTS-Os4CL(O),

Table 1 Plasmids, bacterial strains, and primers used in this study

Plasmids/Strains	Description	Sources or reference
Plasmids		
pACYCDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CloDE13 ori, Str^r	Novagen
PA-SeTAL	pACYCDuet carrying TAL from *Saccharothrix espanaensis*	
PC-Pc4CL-VvSTS(P)	pCDFDuet carrying Pc4CL from *Petroselinum crispum* and VvSTS from *Vitis vinifera*. The genes are regulated by the respective T7 promoter	This study
PC-Sc4CL-VvSTS(P)	pCDFDuet carrying Sc4CL from *Streptomyces coelicolar* A(3) and VvSTS from *Vitis vinifera*. The genes are regulated by the respective T7 promoter	This study
PC-Os4CL-VvSTS(P)	pCDFDuet carrying Os4CL from *Oryza sativa* and VvSTS from *Vitis vinifera*. The genes are regulated by the respective T7 promoter	This study
PC-At4CL-VvSTS(P)	pCDFDuet carrying At4CL from *Arabidopsis thaliana* and VvSTS from *Vitis vinifera*. The genes are regulated by the respective T7 promoter	This study
PC-Le4CL-VvSTS(P)	pCDFDuet carrying Le4CL from *Lithospermum erythrorhizon* and VvSTS from *Vitis vinifera*. The genes are regulated by the respective T7 promoter	This study
PC-Os4CL-AhSTS(P)	pCDFDuet carrying Os4CL from *Oryza sativa* and AhSTS from *Arachis hypogaea*. The genes are regulated by the respective T7 promoter	This study
PC-Os4CL-PaSTS(P)	pCDFDuet carrying Os4CL from *Oryza sativa* and PaSTS from *Picea abies*. The genes are regulated by the respective T7 promoter	This study
PC-AhSTS-Os4CL(O)	pCDFDuet carrying Os4CL from *Oryza sativa* and AhSTS from *Arachis hypogaea*. The genes are regulated by one T7 promoter	This study
PC-PaSTS-Os4CL(O)	pCDFDuet carrying Os4CL from *Oryza sativa* and PaSTS from *Picea abies*. The genes are regulated by one T7 promoter	This study
PC-VvSTS-Os4CL(O)	pCDFDuet carrying Os4CL from *Oryza sativa* and VvSTS from *Vitis vinifera*. The genes are regulated by one T7 promoter	This study
PA-aroG-SeTAL-tyrA	pACYCDuet carrying TAL from *S. espanaensis*, aroG, and tyrA from *E. coli*	Kim el al. [18]
PA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$	pACYCDuet carrying TAL from *S. espanaensis*, $aroG^{fbr}$, ppsA, tktA, and $tyrA^{fbr}$ from *E. coli*	Kim el al. [18]
Strains		
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	
B-TP	BL21(DE3) *ΔtyrR::FRT-ΔpheA::FRT-*kan^R*-FRT*	Kim et al. [18]
B-TPFI	BL21(DE3) *ΔtyrR::FRT-ΔpheA::FRT-ΔfumC::FRT-ΔicdA::FRT-*kan^R*-FRT*	This study
BP-Pc4CL	BL21(DE3) harboring PC-Pc4CL-VvSTS(P)	This study
BP-Os4CL	BL21(DE3) harboring PC-Os4CL-VvSTS(P)	This study
BP-At4CL	BL21(DE3) harboring PC-At4CL-VvSTS(P)	This study
BP-Sa4CL	BL21(DE3) harboring PC-Sa4CL-VvSTS(P)	This study
BP-Le4CL	BL21(DE3) harboring PC-Le4CL-VvSTS(P)	This study
BP-AhSTS	BL21(DE3) harboring PC-Os4CL-AhSTS(P)	This study
BP-PaSTS	BL21(DE3) harboring PC-Os4CL-PaSTS(P)	This study
BO-VvSTS	BL21(DE3) harboring PC-Os4CL-VvSTS(O)	This study
BO-AhSTS	BL21(DE3) harboring PC-Os4CL-AhSTS(O)	This study
BO-PaSTS	BL21(DE3) harboring PC-Os4CL-PaSTS(O)	This study
BL101	BL21(DE3) harboring PA-SeTAL and PC-VvSTS-Os4CL(O)	This study
BL102	BL21(DE3) harboring PA-aroG-SeTAL-tyrA and PC-VvSTS-Os4CL(O)	This study
BL103	BL21(DE3) harboring PA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$ and PC-VvSTS-Os4CL(O)	This study
BTP-S	BTP harboring PA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$ and PC-VvSTS-Os4CL(O)	This study
BTPFI-S	BTPFI harboring PA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$ and PC-VvSTS-Os4CL(O)	This study
Primers		
tyrR-F-Del	GTGTCATATCATCATATTAATTGTTCTTTTTTCAGGTGAAGGTTCCCATGaattaaccctcactaaa-gggcg	
tyrR-R-Del	TTGCACCATCAGGCATATTCGCGCTTACTCTTCGTTCTTCTTCTGACTCAtaatacgactcac-tatagggctc	
pheA-F-Del	CCTCCCAAATCGGGGGCCTTTTTTATTGATAACAAAAAGGCAACACTATGaattaaccctcactaaa-gggcg	

Table 1 (continued)

Plasmids/Strains	Description	Sources or reference
pheA-F-Del	CACATCATCCGGCACCTTTTCATCAGGTTGGATCAACAGGCACTACGTTCtaatacgactcac-tatagggctc	
fumC-F-del	TTAACGCCCGGCTTTCATACTGCCGACCATCTGTTCTGGCCGTACCCAGCaattaaccctcactaaa-gggcg	
fumC-R-del	ATGAATACAGTACGCAGCGAAAAAGATTCGATGGGGGCGATTGATGTCCCtaatacgactcac-tatagggctc	
icdA-F-del	ATGGAAAGTAAAGTAGTTGTTCCGGCACAAGGCAAGAAGATCACCCTGCAaattaaccctcactaaa-gggcg	
icdA-R-del	TTACATGTTCTTGATGATCGCATCACCAAATTCTGAACATTTCAGCAGTTtaatacgactcac-tatagggctc	
Os4CL-*Bam*HI	ATggatccGATGGGGTCGGTGGCGGCGG	
Os4CL-*Not*I	ATgcggccgcTTAGCTGCTTTTGGGCGC	
Os4CL2-*Hind*III-RBS	ATaagctt*AGGAGGATTACAAA*ATGATCACGGTGGCG	
Os4CL2-*Not*I	ATgcggccgcTCAGCACGCCGCGAGCTTGGCT	
Sc4CL-*Bam*HI	AAggatccGATGTTCCGCAGCGAGTACGCA	
Sc4CL-*Not*I	AAgcggccgcTCATCGCGGCTCCCTGAGCT	
Le4CL-*Bam*HI	ATggatccGATGGACACTCAAACCAAAA	
Le4CL-*Not*I	CAgcggccgcTAATTGTGTACACCATTTG	
Pc4CL-*Eco*RI	ATgaattcATGGGAGACTGTGTAGCACC	
Pc4CL-*Not*I	ATgcggccgcTTATTTGGGAAGATCACCGG	
VvSTS-*Eco*RI	ATgaattcGATGGCTTCAGTCGAGGAATTT	
VvSTS-*Not*I	ATgcggccgcTTAATTTGTAACCATAGGAA	
VvSTS-*Hind*III	GCaagcttTTAATTTGTAACCATAGGAA	
VvSTS-*sma*I	ATcccgggGATGGCTTCAGTCGAGGAATTT	
VvSTS-*Xho*I	ATctcgagTTAATTTGTAACCATAGGAATG	
AhSTS-*Eco*RV	ATgatatcGATGGTGTCTGTGAGTGGAATT	
AhSTS-*Xho*I	CAtctcgaGTTATATGGCCACACTGCGGAG	
AhSTS-*Eco*RV	ATgatatcGATGGTGTCTGTGAGTGGAATT	
AhSTS-*Xho*I	CATctcgagTTATATGGCCACACTGCGGAG	
AhSTS-*Eco*RI	ATgaattcGATGGTGTCTGTGAGTGGAATCCGCA	
AhSTS-*Hind*III	ATaagcttTTATATGGCCACACTGCGGAGA	
AhSTS-*Xba*I	ATtctagaTTATATGGCCACACTGCGGAGAAC	
AhSTS-HindIII silencing-F	TGACGCGCTCAATAAgGCTTTTGATCCATTG	
AhSTS-HindIII silencing-R	CAATGGATCAAAAGCCTTATTGAGCGCGTCA	
PsSTS-*Eco*RV	ATgatatcGATGTCTGCAGGAATGACTGTT	
PaSTS-*Sal*I	ATgtcgacTCATGGAAGGAGAACGCTCTTA	
PsSTS-*Eco*RI	ATgaattcGATGTCTGTAGGAATGGGCGTTGATTTGGAGGCTTTCAGGAA	
PsSTS-*Hind*III	ATaagcttTCATGGAAGGAGAACGCTCTTA	
SeTAL-aflII	atcttaagTCATCCGAAATCCTTCCCGTC	
T7-SeTAL-*Not*I	ATgcggccgcGGATCTCGACGCTCTCCCTTAT	
aroG-*Eco*RI	ATgaattcgATGAATTATCAGAACGACGAT	
aroG-*Sal*I	ATgtcgacTTACCCGCGACGCGCTTTTACT	
tyrA-*Nde*I	ATcatatgATGGTTGCTGAATTGACCGCAT	
tyrA-*Kpn*I	CATggtaccTTACTGGCGGTTGTCATTCGC	
ppsA-RBS-*Xho*I	ATctcgagAGGAGGCCATCCATGTCCAACAATGGCTCGTC	
ppsA-*Sal*I-*Not*I	ATgcggccgcGCTgtcgacTTATTTCTTCAGTTCAGCCAG	
tktA-RBS-*Xho*I	ATctcgagAGGAGGCCATCCATGTCCTCACGTAAAGAGCT	
tktA-*Not*I	CATgcggccgcTTACAGCAGTTCTTTTGCTTTC	

PC-VvSTS-Os4CL(O), and PC-PaSTS-Os4CL(O) contained a single promoter but an RBS site in front of each gene.

The aroG, tyrA, and feedback resistance mutants of aroG (aroG^{fbr}) and tyrA (tyrA^{fbr}) were cloned in our previous study [18]. Briefly, to make the pA-aroG^{fbr}-ppsA-tktA-SeTAL-tyrA^{fbr} construct, *aroG^{fbr}* and *tyrA^{fbr}* were introduced into the *Eco*RI and *Sal*I sites and the *Nde* I and *Kpn*I sites of pACYCDuet, respectively, and was named PA-aroG^{fbr}-tyrA^{fbr}. *ppsA* (phosphoenolpyruvate synthase) was amplified using PCR with the forward primer including an RBS and *Xho*I site, and a reverse primer containing *Sal*I and *Not*I sites. The resulting PCR product was digested with the corresponding restriction enzymes and cloned into the *Sal*I and *Not*I sites of PA-aroG^{fbr}-tyrA^{fbr} and named PA-aroG^{fbr}-ppsA-tyrA^{fbr}. *tktA* (Transketolase 1) was amplified using PCR with the forward primer containing an RBS and *Xho*I site and the reverse primer containing a *Not*I site. The resulting PCR product was digested with the corresponding restriction enzymes and cloned into the *Sal*I and *Not*I sites of PA-aroG^{fbr}-tyrA^{fbr} and named PA-aroG^{fbr}-ppsA-tktA-tyrA^{fbr}. *SeTAL* containing the T7 promoter and RBS was amplified with two primers expanded with the *Not*I and *afl*II sites using PA-SeTAL as a template. The PCR product was digested with *NotI* and *afl*II restriction enzymes. The resulting DNA fragment was cloned into the corresponding site of PA-aroG^{fbr}-ppsA-tktA-tyrA^{fbr} and named PA-aroG ^{fbr}-ppsA-tktA-SeTAL-tyrA^{fbr} (Table 1).

Deletion of Δ*tyrR*, Δ*pheA*, Δ*icdA*, and Δ*fumC*

The Quick and Easy Conditional Knockout Kit (Gene Bridges, Heidelberg, Germany) was used to prepare *E. coli* BL21 (DE3) mutants of four genes, *tyrR* (DNA-binding transcriptional dual regulator), *pheA* (chrismate mutase/prephenate dehydratase), *icdA* (isocitrate dehydrogenase), and *fumC* (fumarase C). Briefly, the *tyrR* gene of *E. coli* BL21 (DE3) was deleted using tyrR-FRT-PGK-gb2-neo-FRT-tyrR cassette generated through PCR using two primers containing the *tyrR*-specific sequence of 50 bp, with FRT-PGK-gb2-neo-FRT as a template (Table 1). Luria–Bertani (LB) agar plates supplemented with 50 μL/mL kanamycin were used for the selection of positive colonies. The positive clones of *tyrR* deletion were checked using colony PCR. The positive clone selected was named BL21Δ*tyrR*. To make Δ*tyrR* and Δ*pheA* double mutants, BL21Δ*tyrR* was used. A 708-FLPe expression plasmid encoding FLPe recombinase was used to remove the kanamycin cassette in BL21Δ*tyrR*. Colony PCR was used to verify the positive clone. The BL21Δ*tyrR* strain, a kanamycin cassette-free, was used to remove *pheA* using pheA-FRT-PGK-gb2-neo-FRT-pheA cassette generated through PCR. The double deletion mutant of *tyrR* and *pheA* was designated as BL21Δ*tyrR-pheA*. The quadruple mutant, BL21Δ*tyrR-pheA-fumC-icdA*, was generated by repeating the same methods as described above.

Production of resveratrol from glucose and glycerol in *E. coli*

To compare the effects of different constructs on the production of resveratrol, each construct was transformed into *E. coli* BL21 (DE3). Each *E. coli* transformant was grown overnight in LB broth containing the appropriate antibiotics at a concentration of 50 μg/mL. The overnight cultured cells were transferred into fresh LB medium containing appropriate antibiotics, and the cells were grown until the OD_{600} reached 1.0. The cells were collected through centrifugation and then washed briefly with the M9 medium. The cell concentration was controlled to an OD_{600} of 1.0, with 2 mL of M9 medium supplemented with 1% glucose or 1% glycerol, 1% yeast extract, and 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The resulting culture was incubated at 30 °C for 48 h with shaking at 200 rpm. Samples (200 μL) were collected and mixed with 1 mL of ethyl acetate. After vortexing and centrifugation, the upper ethyl acetate layer was transferred into a new 1.5 mL Eppendorf tube. The organic layer was evaporated to dryness, and the remaining residues were dissolved in 100 μL of dimethyl sulfoxide (DMSO). The samples were analyzed using a Varian HPLC (High performance liquid chromatography) system equipped with a photodiode array detector and an Agilent Polaris 5 C18-A column (250 × 4.6 mm) was used for the analysis of the reaction products using 20 μL injection. The mobile phases consisted of 0.1% formic acid in water or acetonitrile. The program was 20% acetonitrile at 0 min, 45% acetonitrile at 10 min, 80% acetonitrile at 20 min, 90% acetonitrile at 20.1 min, 90% acetonitrile at 25 min, 20% acetonitrile at 25.1 min, and 20% acetonitrile at 30 min. The flow rate was 1 mL/min, and UV detection for the reaction products was dually monitored at 270 and 310 nm.

Results and discussion

Production of resveratrol in *E. coli* from *p*-coumaric acid

To synthesize resveratrol from glucose in *E. coli*, at least three genes, namely *TAL*, *4CL*, and *STS* are necessary (Fig. 1). The TAL gene is mainly found in microorganisms, and the TAL gene of *Saccharothrix espanaensis*, which has been widely used in previous studies, was used in this study [18, 36]. Two genes, *4CL* and *STS*, are more important for resveratrol production. Therefore, we decided to find the best combination of *4CL* and *STS* for resveratrol production from *p*-coumaric acid in *E. coli* BL21 (DE3). The 4CL gene is found simultaneously

in microorganisms and plants. To select most effective genes for resveratrol biosynthesis, we tested four *4CL* genes, *Pc4CL*, *Os4CL,* and *Le4CL* from plants and *Sc4CL* from micororganism, through combination with *VvSTS* for resveratrol production (Fig. 2). Four different combinations of *4CLs* and *VvSTS*, which are regulated by independent T7 promoter, were tested for resveratrol production from *p*-coumaric acid (Fig. 2a). BP-Os4CL harboring *Os4CL* and *VvSTS* showed the highest productivity of 16.2 mg/L after 12 h of incubation, followed by BP-Pc4CL containing *Pc4CL* and *VvSTS* (8.1 mg/L). However, Strains containing *Sc4CL* and *VvST* (BP-Sc4CL) or *Le4C*L and *VvSTS* (BP-Le4CL) produced resveratrol of less than 4 mg/L. Based on the above results, we decided to use *Os4CL* in subsequent experiments. Next, we tested six different combinations of three STSs (*VvSTS, AhSTS*, and *PaSTS*) and *Os4CL*, which were constructed either pseudo-operon or operon regulated by the T7 promoter for resveratrol production from *p*-coumaric acid in *E. coli* (Fig. 2b). *E. coli* harboring *Os4CL* and *VvSTS* pseudo-operon (BP-VvSTS) regulated by each T7 promoter or *Os4CL* and *VvSTS* operon (BO-VvSTS) regulated by one T7 promoter, showed the highest productivity at 15.1 and 19.8 mg/L after 24 h of incubation, respectively. Interestingly, BO-AhSTS strain containing *the AhSTS* and *Os4CL* operon produced only small amounts of resveratrol, whereas BP-AhSTS strain containing *the AhSTS* and *Os4CL* pseudo-operon produced 13.8 mg/L of resveratrol. *E. coli* containing either *PaSTS* and *Os4C*L pseudo-operon (BP-PaSTS) or *PaSTS* and *Os4CL* operon (BO-PaSTS) produced only small amounts of resveratrol (less than 4 mg/L). These results indicate that the optimum combination of *STS* and *4CL* for balanced gene expression is critical for the production of resveratrol. We chose the VvSTS-Os4CL(O) operon (*E. coli* strain BO-VvSTS) for further experiments.

Production of resveratrol from glucose in *E. coli*

Resveratrol biosynthesis in plants begins with cinnamic acid catalyzed by PAL with phenylalanine as the substrate (Fig. 1). Cinnamic acid is catalyzed by C4H to form *p*-coumaric acid [34]. Since C4H is a member of the P450 family, it is difficult to express it functionally in *E. coli*. For this reason, many studies related to the biosynthesis of phenolic compounds have used TAL, which converts tyrosine to *p*-coumaric acid [18, 35]. In previous studies, *TAL* cloned from *S. espanaensis* was used to biosynthesize various phenolic compounds in *E. coli* based on its excellent enzyme activity [18, 36]. pA-SeTAL was transformed into the *E. coli* BL 100 strain, and the resulting strain, BL 101, was examined for the production of resveratrol from glucose. After a 24 h incubation, HPLC analysis of the reaction products showed two new peaks at 8.82 and 10.29 min. Based on the HPLC retention

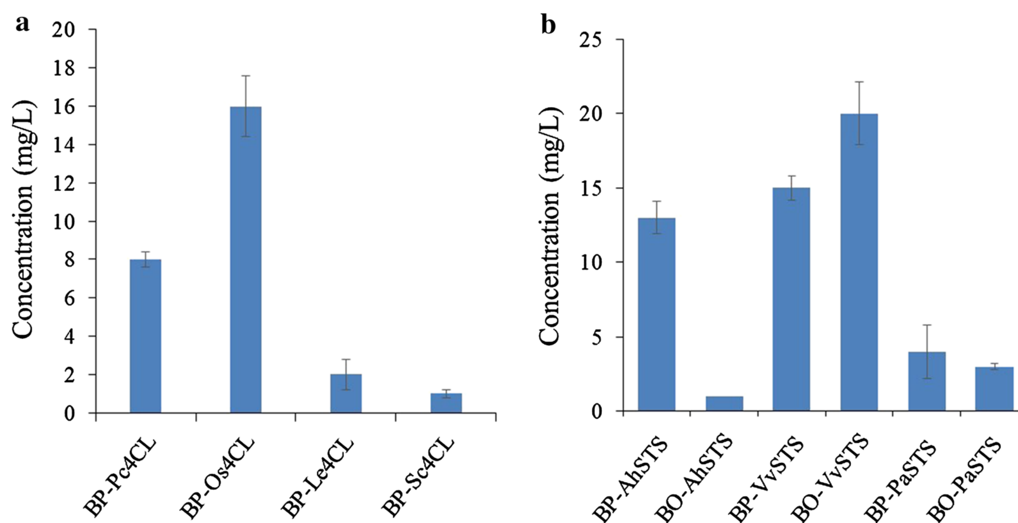

Fig. 2 Effect of different 4CL genes (**a**) and STS genes (**b**) on the production of resveratrol. After 12 h of induction at 25 °C, the cells were collected through centrifugation and resuspended in 2 mL of M9 containing 1% yeast extract and 2% glucose. The cell density was adjusted to OD_{600} = 3.0. p-Coumaric acid (200 μM) was added and the cells were cultured at 30 °C. The culture was stopped after 6 h by adding two volumes of ethyl acetate. The reaction products were analyzed using HPLC. The error bars indicate mean values ± from three independent experiments. BP-Pc4CL, BL21(DE3) harboring PC-Pc4CL-VvSTS(P); BP-Os4CL, BL21(DE3) harboring PC-Os4CL-VvSTS(P); BP-At4CL, BL21(DE3) harboring PC-At4CL-VvSTS(P); BP-Sa4CL, BL21(DE3) harboring PC-Sa4CL-VvSTS(P); BP-Le4CL, BL21(DE3) harboring PC-Le4CL-VvSTS(P); BP-AhSTS, BL21(DE3) harboring PC-Os4CL-AhSTS(P); BP-PaSTS, BL21(DE3) harboring PC-Os4CL-PaSTS(P); BO-VvSTS,BL21(DE3) harboring PC-Os4CL-VvSTS(O); BO-AhSTS,BL21(DE3) harboring PC-Os4CL-AhSTS(O); BO-PaSTSBL21(DE3) harboring PC-Os4CL-PaSTS(O)

time, molecular mass, and UV absorbance, the two peaks at 8.82 and 10.29 min turned out to be *p*-coumaric acid and resveratrol, respectively (Fig. 3a–c). To further clarify the structure of P2, MS (Mass spectrometry) and NMR (Nuclear Magnetic Resonance) analyses were performed. In the negative ESI mode, P2 showed a molecular ion $[M-H]^-$ at *m/z* 227, which was consistent with the molecular weight of resveratrol (Fig. 3d–f). For NMR analysis, one liter was cultured, the reaction product was extracted twice with the same amount of ethyl acetate, purified first using TLC, and then finally purified using HPLC and the same method as the analysis conditions. Finally, 5 mg of the pure reaction product was obtained and used for ^{1}H-NMR analysis. NMR was conducted as described by Kim et al. [37]. The structure of the reactants was determined by comparison with the structure published by Amalfitano et al. [38].

The NMR data was analyzed as follows: ^{1}H-NMR (400 MHz, Acetone-d_6); δ 7.41 (2H, *d*, 8.58 Hz), δ 7.01(1H, *d*, 16.3 Hz), δ 6.88(1H, *d*, 16.3 Hz), δ 6.83(2H, d, 8.6 Hz), δ 6.5 (2H, *d*, 2.11 Hz), and δ 6.26(1H, *t*, 2.11 Hz). The results were consistent with previously published results [38]. These results indicated that resveratrol was successfully synthesized from glucose in strain BL101 harboring *SeTAL*, *VvSTS*, and *Os4CL*, with a yield of 13.8 mg/L (Fig. 4a). The production of BL101 (13.8 mg/L) was lower than that of *E. coli* strain BO-VvSTS (19.8 mg/L). This result is most likely due to insufficient supply of endogenous tyrosine in *E. coli* for resveratrol biosynthesis. Therefore, we decided to increase the supply of tyrosine, which is used as a precursor for *p*-coumaric acid biosynthesis.

Engineering of *E. coli* to increase the production of resveratrol

Although resveratrol was biosynthesized from glucose using the wild type *E. coli* strain containing three genes, the yield was not high. This was due to the limited supply of tyrosine. Therefore, we decided to increase the amino acid and tyrosine pool, which is the TAL substrate for *p*-coumaric acid biosynthesis, which was used as an entry point for resveratrol biosynthesis. To increase tyrosine levels, we overexpressed *aroG* and *tyrA*, which encodes the rate-limiting enzymes in the shikimic acid biosynthetic pathway [17, 18]. The aroG is converted into 3-deoxy-d-arabinoheptulosonate-7-phosphate using both phosphoenolpyruvate and erythrose 4-phosphate as substrates. tyrA is responsible for a two-step reaction from chorismate via prephenate to 4-hydroxy-phenylpyruvate. Additionally, two genes, *aroG*^{fbr} and *tyrA*^{fbr},

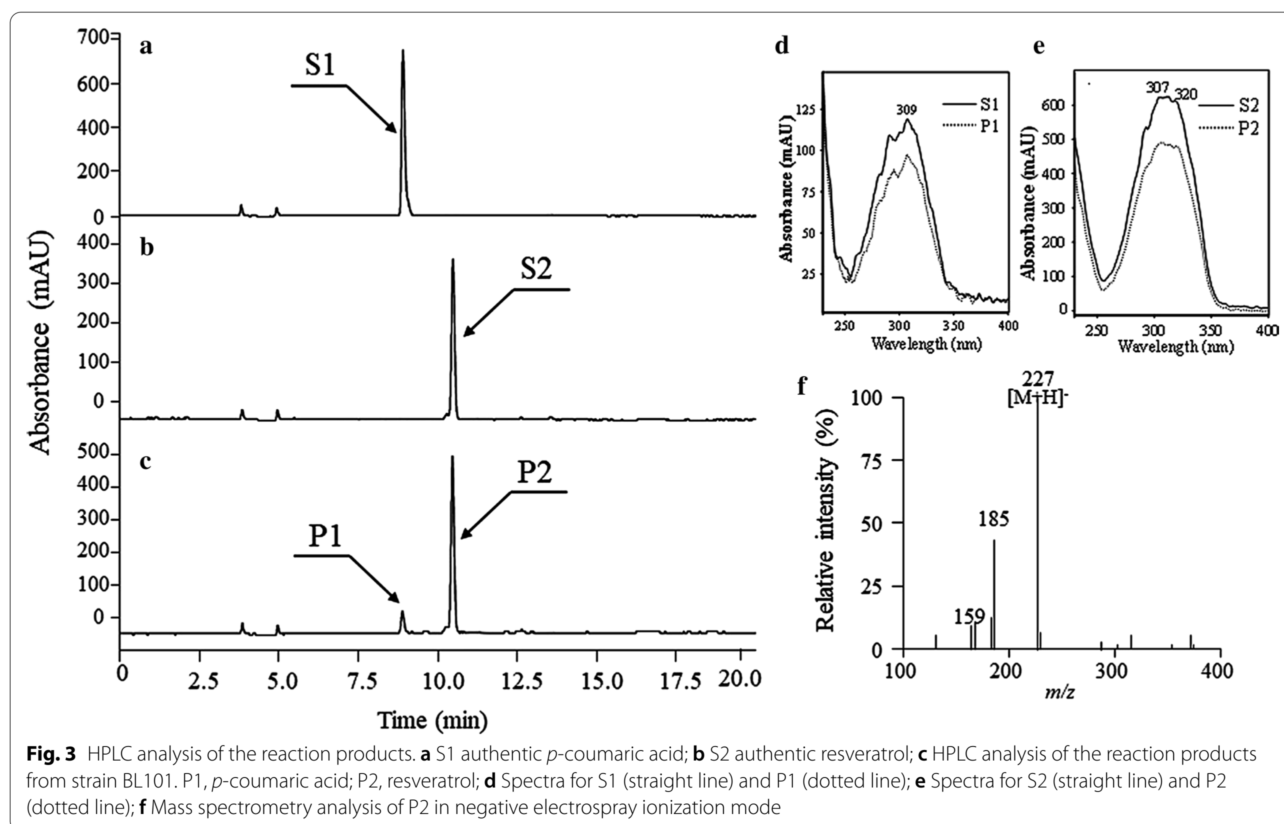

Fig. 3 HPLC analysis of the reaction products. **a** S1 authentic *p*-coumaric acid; **b** S2 authentic resveratrol; **c** HPLC analysis of the reaction products from strain BL101. P1, *p*-coumaric acid; P2, resveratrol; **d** Spectra for S1 (straight line) and P1 (dotted line); **e** Spectra for S2 (straight line) and P2 (dotted line); **f** Mass spectrometry analysis of P2 in negative electrospray ionization mode

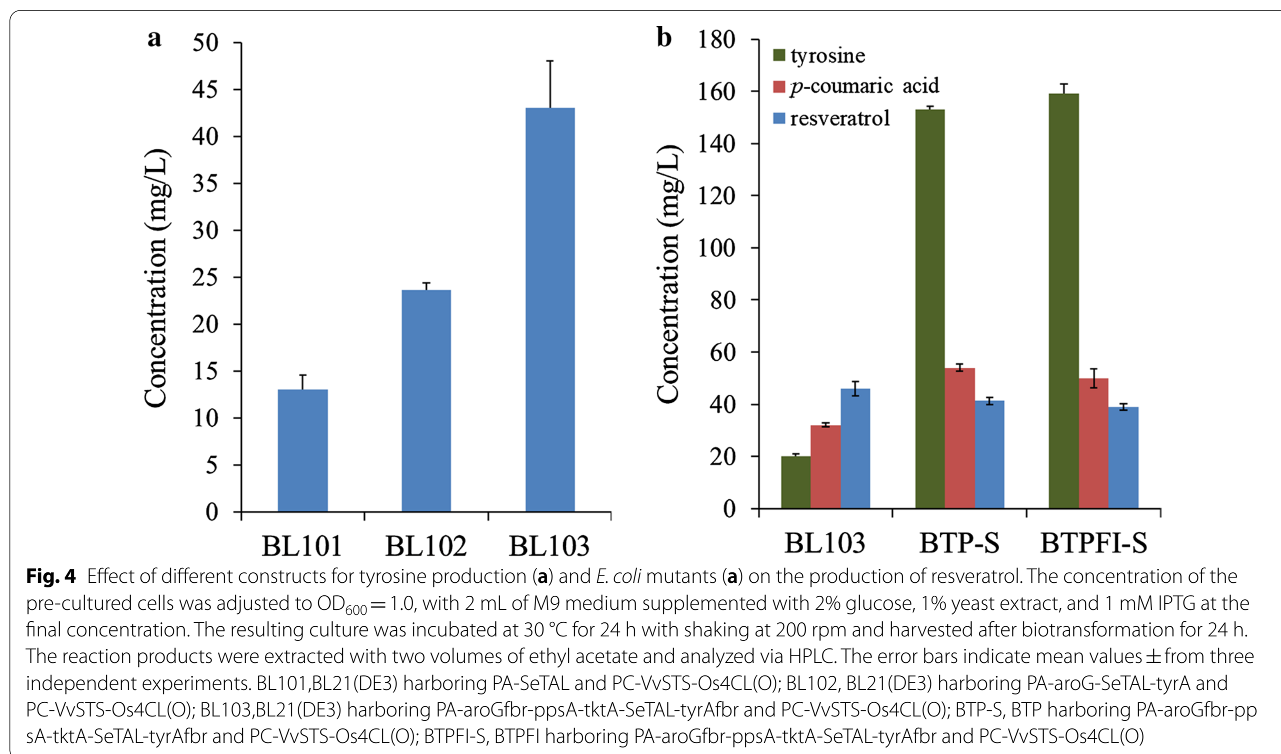

Fig. 4 Effect of different constructs for tyrosine production (**a**) and *E. coli* mutants (**a**) on the production of resveratrol. The concentration of the pre-cultured cells was adjusted to $OD_{600} = 1.0$, with 2 mL of M9 medium supplemented with 2% glucose, 1% yeast extract, and 1 mM IPTG at the final concentration. The resulting culture was incubated at 30 °C for 24 h with shaking at 200 rpm and harvested after biotransformation for 24 h. The reaction products were extracted with two volumes of ethyl acetate and analyzed via HPLC. The error bars indicate mean values ± from three independent experiments. BL101,BL21(DE3) harboring PA-SeTAL and PC-VvSTS-Os4CL(O); BL102, BL21(DE3) harboring PA-aroG-SeTAL-tyrA and PC-VvSTS-Os4CL(O); BL103,BL21(DE3) harboring PA-aroGfbr-ppsA-tktA-SeTAL-tyrAfbr and PC-VvSTS-Os4CL(O); BTP-S, BTP harboring PA-aroGfbr-ppsA-tktA-SeTAL-tyrAfbr and PC-VvSTS-Os4CL(O); BTPFI-S, BTPFI harboring PA-aroGfbr-ppsA-tktA-SeTAL-tyrAfbr and PC-VvSTS-Os4CL(O)

were overexpressed because aroG and tyrA are feedback inhibited by tyrosine. $aroG^{fbr}$, a mutant form of aroG, is not inhibited by the end product, tyrosine, unlike aroG (Fig. 1). In addition, tyrA was changed to $tyrA^{fbr}$, which is not inhibited by the end product, tyrosine (Fig. 1) [17, 18]. Two genes, *ppsA* and *tktA*, which were overexpressed are involved in the biosynthesis of phosphoenolpyruvate and erythrose-4-phosphate, which are used as entrance compounds for the shikimic acid biosynthetic pathway. *E. coli* BL21(DE3) was used as the host strain for resveratrol production using three different vector sets, pA-SeTAL (BL101), pA-aroG-SeTAL (BL102), and pA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$ (BL103), along with the PC-VvSTS-Os4CL operon. The resulting three *E. coli* strains were compared for resveratrol production. BL103 containing pA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$ produced a much greater amount (42.9 mg/L) of resveratrol, followed by strain BL102 (23.6 mg/L) (Fig. 4a). These results indicate that the increased supply of tyrosine in *E. coli* had a great effect on the biosynthesis of resveratrol. Thus, we decided to increase the tyrosine supply in *E. coli* by the deletion of two genes, *tyrR* and *pheA*, which have been reported to increase tyrosine levels in several studies. *tyrR* encodes a transcriptional regulatory protein for tyrosine biosynthesis. Its transcription is regulated by the feedback inhibition of the end product, tyrosine. In previous studies, the deletion of *tyrR* in *E. coli* increased tyrosine production [18]. Because both tyrA and pheA compete to use prephenate as a substrate, *pheA* was deleted to induce tyrosine biosynthesis. Next, the removal of the *icdA* and *fumC* genes encoding isocitrate dehydrogenase and fumarate hydratase, respectively, has been reported to increase the amount of CoA in *E. coli* (Fig. 1) [18]. Therefore, the two genes were selected for engineering. This was expected to increase the biosynthesis of *p*-coumaroyl-CoA, which is used as an intermediate metabolite of resveratrol biosynthesis. As a result, three strains, BL21 DE3(wild type), B-TP (tyrA/pheA double mutant), and B-TPFI (*tyrA*/*pheA*/*icdA*/*fumC* quadruple mutant) were used to create three different strain sets along with two different vector sets, pA-$aroG^{fbr}$-ppsA-tktA-SeTAL-$tyrA^{fbr}$ and PC-VvSTS-Os4CL operon. The three strains were named BL103, BTP-S, and BTPFI-S, and resveratrol production was compared for each strain. BTP-S and BTPFI-S produced 41.3 mg/L and 38.9 mg/L, respectively, which was less than the yield of BL103 (50.3 mg/L) (Fig. 4b). In both the BTP-S and BTPFI-S strains, more than 150 mg/L of tyrosine was detected, whereas in the wild type, 21.7 mg/L of tyrosine was detected (Fig. 4b). In addition, BTP-S (54.3 mg/L) and BTPFI-S (50.1 mg/L) accumulated more *p*-coumaric acid than BL103 (32.1 mg/L). These results show that both tyrosine and *p*-coumaric acid increased in the mutant strains, but resveratrol did not. It seemed that the extra tyrosine or *p*-coumaric acid

might inhibit the next two steps, *p*-coumaroyl-CoA synthesis or resveratrol synthesis. This resulted in a lower final yield of resveratrol in the mutant strains than in the wild type. Therefore, when resveratrol is synthesized from simple carbon sources, such as glucose and glycerol, balancing between the precursor and the product is critical for increasing the final yield. Recently, various methods, such as the co-culture methods, introduction of promoters of different strengths, and gene integration into the genome, have been attempted to resolve the metabolic imbalances. Among them, a co-culture method that divides the metabolic pathway necessary for the biosynthesis of phytochemicals into two or three strains has been frequently used as a method to overcome metabolic imbalance [35, 39]. To increase resveratrol biosynthesis, subsequent research such as a co-culture method should be conducted.

We explored the optimum culture method for resveratrol production using BL 103. First, we optimized the culture temperature. The cells were cultured at 25, 30, and 37 ℃. The production of resveratrol was the highest with 44.3 mg/L at 30 °C, followed by 23.1 mg/L at 25 ℃ and 3.2 mg/L (Fig. 5a). Although the production of resveratrol was the lowest at 37 ℃, the production of

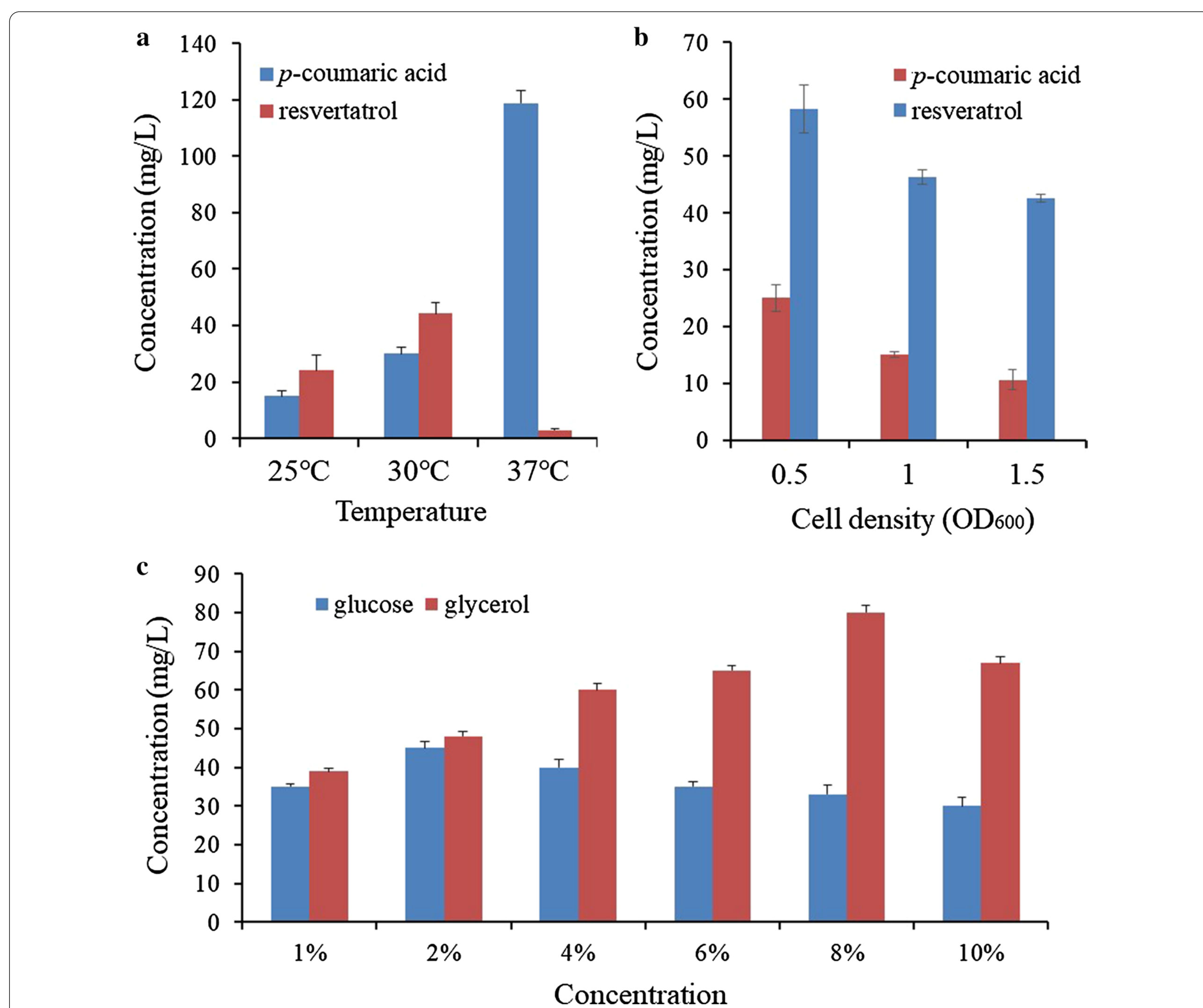

Fig. 5 Effect of biotransformation temperature (**a**), cell density (**b**), and different kinds and concentrations of carbon source (**c**) on the production of resveratrol. The concentration of the pre-cultured cells was adjusted to $OD_{600} = 1.0$, with 2 mL of M9 medium supplemented with 2% glucose, 1% yeast extract, and 1 mM IPTG at the final concentration for A and B. C was performed in the same manner as above, except for the carbon source. The resulting culture was incubated at 30 °C for 24 h with shaking at 200 rpm and harvested after biotransformation for 24 h. The reaction products were extracted with two volumes of ethyl acetate and analyzed via HPLC. The error bars indicate mean values ± from three independent experiments

p-coumaric acid was the highest at 118.9 mg/L among the temperatures tested (Fig. 5a). These results suggest that the production of high amounts of proteins such as 4CL and STS may form inclusion bodies. The cells grown at 25 ℃ produced a lower amount of *p*-coumaric acid and resveratrol than those at 30 ℃. These results suggest that the cells cultivated at 25 ℃ did not produce enough proteins for resveratrol production owing to the low culture temperature. We also optimized cell culture density. The cell density was adjusted to 0.5, 1, 1.5, and 2 at OD_{600}. The optimal cell density was 0.5 at OD_{600}, at which approximately 58.7 mg/L resveratrol was produced (Fig. 5b). However, as cell density increased, the production of resveratrol decreased. Next, we determined the optimal carbon sources and supply concentrations. The cell densities were adjusted to 0.5 at OD_{600} and glucose and glycerol were supplied at a rate of 1, 2, 4, 6, 8, and 10%. Overall, the productivity of resveratrol was higher when glycerol was used as a carbon source than glucose. When glycerol was used as a carbon source, the productivity of resveratrol increased as the supply concentration increased. The highest productivity of resveratrol was observed at a concentration of 8%, at which approximately 78.9 mg/L of resveratrol was produced (Fig. 5c). In the case of glucose, the productivity was highest at a concentration of 2%, at which approximately 45.4 mg/L of resveratrol was produced. However, as the concentration of glucose increased, the productivity of resveratrol gradually decreased.

Subsequently, resveratrol production from glycerol using the optimized conditions was monitored for 48 h. *p*-Coumaric acid was observed after 8 h of incubation and it slightly increased with incubation time. The cell density gradually increased with incubation time. The highest density was observed after 24 h of incubation, at which time the cell density reached approximately 7.5 at OD_{600}. After 24 h incubation, the cell density showed a tendency to gradually decrease. Resveratrol production was initiated after 8 h of incubation and it rapidly increased until 24 h of incubation. At this time, approximately 68.9 mg/L resveratrol was produced. After 24 h of incubation, resveratrol production slowly increased. After 40 h of incubation, resveratrol production was highest at 80.4 mg/L and then it slightly decreased (Fig. 6). Wang et al. [28] reported the biosynthesis of 114.4 mg/L of resveratrol from glucose, which was 1.4 times higher than that of this study. However, in the study of Wang et al. [28], resveratrol was biosynthesized by adding 3 mM tyrosine to the culture medium, but we synthesized resveratrol from glucose without supplying tyrosine to the medium. For commercial application of resveratrol, a yield of at least 1.0 g/L must be reached. However, the yield for this study

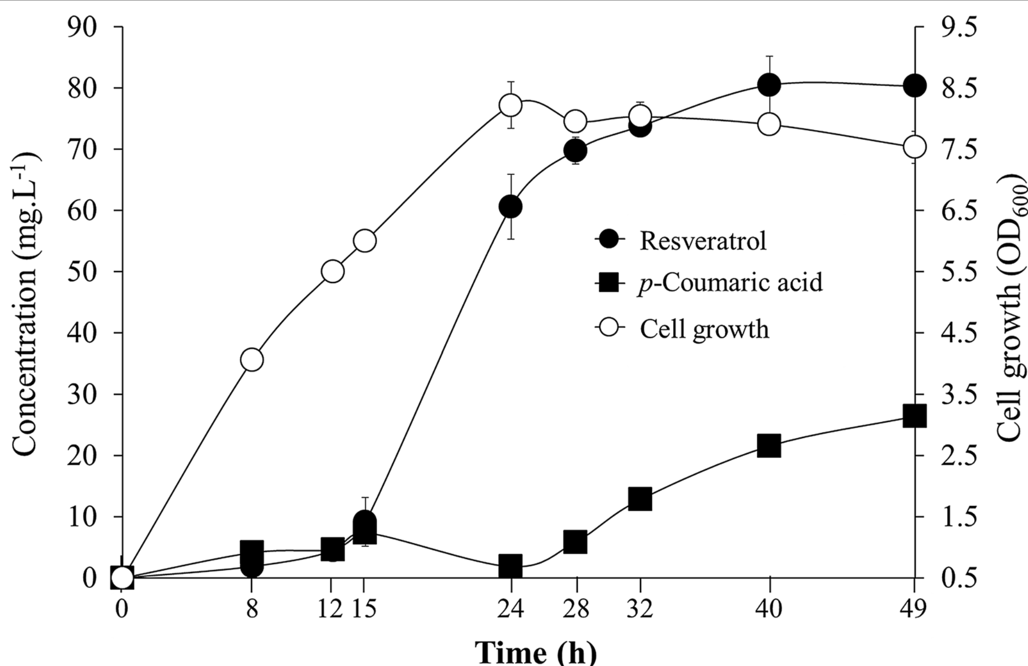

Fig. 6 Production of resveratrol by *E. coli* strain BL103. The concentration of the pre-cultured cells was adjusted to $OD_{600} = 0.5$, with 25 mL of M9 medium supplemented with 8% glycerol, 1% yeast extract, and 1 mM IPTG at the final concentration. The flask was incubated at 30 °C for 49 h with shaking at 200 rpm, and the sample was periodically collected. The reaction products were extracted with two volumes of ethyl acetate and analyzed via HPLC. The error bars indicate mean values ± from three independent experiments

is 80.4 mg/L. Of course, it is difficult to apply commercially with the current production amount, but if the culture conditions are optimized using a fermentor and the pathway for resveratrol biosynthesis is slightly improved, it will be possible to produce a sufficient amount required for commercial production.

Conclusion

Resveratrol is attracting much attention due to its various health benefits such as anticancer, antileukemic, and immune-modulating activities [12, 13]. Since resveratrol is supplied by extraction from plants [10, 11], researchers recently attempted to biosynthesize resveratrol by introducing the resveratrol biosynthesis gene into microorganisms as an alternative production method for resveratrol [30–33]. There are several things to consider in order to biosynthesize resveratrol from a simple carbon source using microorganisms. First, resveratrol biosynthetic genes such as *4CL* and *STS* with high turnover rates should be selected. Second, a stable supply of tyrosine, the starting material for resveratrol biosynthesis, must be established. For the stable supply of tyrosine, the metabolic engineering of the tyrosine biosynthetic pathway of microorganisms must be achieved. Third, it is important to balance the metabolic pathways for resveratrol production without the metabolic load of intermediates such as tyrosine, *p*-coumaric acid and p-coumaroyl CoA. In this study, we attempted to produce resveratrol from simple carbon source, considering the problems presented above and optimized the culture system such as cell concentration, culture temperature, and carbon sources. Under optimized conditions, approximately 80.4 mg/L of resveratrol was produced after 48 h of culture using glycerol as a carbon source. Although we have successfully biosynthesized resveratrol from a simple carbon source, we need to further improve the biosynthesis of resveratrol in microorganisms. For this purpose, various methods, such as the co-culture methods, introduction of promoters of different strengths, and gene integration into the genome, must be attempted.

Abbreviations

PAL: Phenylalanine ammonia lyase; *C4H*: Cinnamic acid 4-hydroxylase; *TAL*: Tyrosine ammonia lyase; *4CL*: 4-Coumarate-CoA ligase; *STS*: Stilbene synthase; HPLC: High-performance liquid chromatography; IPTG: Isopropyl-*β*-D-thiogalactoside; LB: Luria broth; *ppsA*: Phosphoenolpyruvate synthetase; *tktA*: Transketolase; *tyrR*: Phenylalanine DNA-binding transcription repressor; *aroG*: Deoxyphosphoheptonate aldolase; aroC: Chorismate synthase; tyrA: Prephenate dehydrogenase; *pheA*: Prephenate dehydratase; *tyrB*: Phenylalanine aminotransferase.

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Authors' contributions

BGK and JHA designed the experiments. JYP, JHL, and BGK performed the experiments and analyzed the data. JYP, JKL, JHA, and BGK wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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