Resveratrol can prevent, or delay, the progression of illnesses such as cancer, cardiovascular disease and ischemic injuries; it can also enhance stress resistance and extend lifespans of various organisms from yeast to vertebrates [2, 7]. These effects have been observed despite its extremely low bioavailability and rapid clearance from the circulation [18]. However, even though resveratrol has therapeutic benefits, its clinical therapeutic effect is limited owing to its low oral bioavailability. An Escherichia coli system was developed that contains an artificial biosynthetic pathway that produces resveratrol glucoside derivatives, such as resveratrol-3-O-glucoside (piceid) and resveratrol-4'-O-glucoside (resveratroloside), from simple carbon sources. This artificial biosynthetic pathway contains a glycosyltransferase addition (YjiC from Bacillus) with resveratrol biosynthetic genes. The produced glucoside compounds were verified through the presence of a product peak(s) and also through LC/MS analyses. The strategy used in this research demonstrates the first harnessing of E. coli for de novo synthesis of resveratrol glucoside derivatives from a simple sugar medium.

**Keywords:** Artificial biosynthesis, resveratrol, glycosylation, piceid

Resveratrol biosynthesis begins with the deamination of phenylalanine through phenylalanine ammonia lyase (PAL) in order to produce cinnamic acid, which is then hydroxylated by cinnamate-4-hydroxylase (C4H) to form p-coumaric acid. This product is attached to coenzyme A (CoA) via 4-coumarate-CoA ligase. Next, stilbene synthase (STS) condenses 4-coumaroyl-CoA, using three molecules of malonyl-CoA to form the resveratrol. Several types of glycosyltransferase have been demonstrated to be involved in the phenylpropanoid metabolism, but there have been
few reports on a specific glycosyltransferase to resveratrol [10, 12]. Although previous studies have already made resveratrol glucoside derivatives in microbe, the approaches were limited to the in vitro enzymatic reaction and the bioconversion of resveratrol by recombinant Escherichia coli [12, 15, 17]. Recently, the YjiC-homologous glycosyltransferases from other Bacillus species have been found to have flexible glycosyltransferase activities toward various benzoic compounds [1, 11, 14, 21]. It was already demonstrated that YjiC glycosyltransferase facilitates the in vitro glycosylation of the aromatic moiety of phenylpropanoid, such as apigenin, phlorectin, and several flavonols [1, 6, 11, 13, 14].

Meanwhile, it has been demonstrated that E. coli can produce resveratrol through the introduction of multiple enzymes in the phenylpropanoid pathway [4]. In this artificial expression system, tyrosine ammonia lyase (TAL) can replace PAL and C4H by producing p-coumaric acid from tyrosine. In this paper, the construction of an artificial biosynthetic pathway containing a UDP-glycosyltransferase gene (yjiC) addition with resveratrol biosynthetic genes is presented (Fig. 1). This E. coli system that contains an artificial biosynthetic pathway produces resveratrol glucoside derivatives, such as piceid (resveratrol-3-O-glucoside) and resveratroloside (resveratrol-4′-O-glucoside), from simple carbon sources. Although these compounds have been previously identified to be produced in various plants, we describe a different approach for their de novo synthesis in E. coli by engineering an artificial biosynthetic pathway.

In order to construct a plasmid that contains the artificial resveratrol glucoside biosynthetic pathway, our previously reported pET-TLkS cloning method was used [4]. The artificial resveratrol biosynthetic plasmids each contained a gene with their own T7 promoter, ribosome-binding site (RBS), and terminator sequence, as in the parental vectors. Although production of the resveratrol in E. coli using precursor (p-coumaric acid) conversion has been previously reported by other researchers [19, 20], the construction of the artificial biosynthetic pathway (pET-TLkS) was first reported as the de novo synthesis of resveratrol in E. coli using a simple sugar medium [4]. Here, the resveratrol-producing construct pET-opTLS was used, as in the parental vectors, that contain the codon-optimized tal gene of Saccharothrix espanaensis [9], cloned cel gene of Streptomyces coelicolor [8], and codon-optimized sts gene of Arachis hypogaea [20]. For the production of resveratrol glucoside derivatives in E. coli, the only addition was the insertion of

![Fig. 1. Engineered biosynthetic pathways for the resveratrol glucoside derivatives starting from tyrosine in E. coli.](image-url)

The artificial resveratrol biosynthetic pathway (pET-opTLS) only produces trans-resveratrol, as demonstrated in the HPLC. However, the cis-resveratrol-3-O-glucoside (cis-piceid) is produced after glycosylation in a long-time culture.
the glycosyltransferase gene (yjiC) in the pET-opTLS plasmid containing the resveratrol biosynthetic pathway (Fig. 2). The DNA fragment containing the promoter, YjiC coding region, and terminator was amplified using the pET302-YjiC plasmid, which is similar to the previously reported YjiC expression vector [13, 21]. The 1.2-kb DNA fragment containing the YjiC coding region was PCR-amplified using primers NSpe (5’-ACTAGTAGGTTGAGGCCGTGTCGACACCGCC-3’) and CSpe (5’-ACTAGTTCCCTCTTTCAGCAAAAAACCCCTC-3’) with pET302-YjiC as a template. The amplified fragments were digested with each restriction enzyme and cloned between the SpeI-digested pET-opTLS, which resulted in pET-opTLYS (Fig. 2).

Recombinant *E. coli* C41(DE3) strain harboring pET-opTLYS was cultured in modified M9 minimal medium supplemented with 15 g/l glucose as the sole carbon source and 25 g/l CaCO$_3$ [9]. Upon 1 mM IPTG induction, the secreted resveratrol glucoside derivatives in the culture medium were analyzed using high-performance liquid chromatography (HPLC) after 36 h. Twenty milliliters of culture was extracted with an equal volume of ethyl acetate. The ethyl acetate was dried in a vacuum, and the dried residue was resuspended in 400 µl of methanol. Twenty microliters of extract was applied to a SunFire C18 column (4.6 × 250 mm, 5 µm; Waters, UK) using a HPLC system (acetonitrile (CH$_3$CN)-H$_2$O (0.05% trifluoroacetic acid) 10% to 40% CH$_3$CN for 40 min, at flow rate of 1 ml/min; Dionex, USA) equipped with a photodiode array detector.
new peaks were reproducibly detected in the engineered strain (Fig. 3). These compounds were further analyzed using liquid chromatography-mass spectrometry (LC-MS) in positive-ion mode. The LC-MS was performed using a LTQ-XL linear ion trap mass spectrophotometer (Thermo Electron, USA) equipped with an electro spray ionization (ESI) source. The HPLC separations were performed using a Dionex 3000 HPLC System unit (Thermo Electron, USA) using a HSS T3 column (2.1 × 150 mm; 2.5 µm; Waters, UK) with a linear gradient of the binary solvent system under similar HPLC conditions as described above. The data-dependent mass spectrometry experiments were controlled using the menu-driven software provided with the Xcalibur system (ver. 2.2 SP1.4; Thermo Scientific, USA). The compounds were identified through comparisons with the standard compounds using the observed retention time, ultraviolet spectra, and mass chromatogram.

The peak 3 at 20.1 min co-eluted with the trans-piceid standard, and they had overlapping UV spectra with two absorbance maxima of 307 and 319 nm (Fig. 3 and 4A(b)). In contrast, a different UV spectrum with an absorbance maximum at 289 nm was obtained for the 24.7 min peak (Fig. 4A(c)). Under a positive ESI mode, the compound eluting at 20.1 min was generated at the m/z 390.92 [M+H]^+ ion. Its identity was confirmed as trans-piceid from the collision-induced dissociation (CID) spectrum (Fig. 4B(b)) of the m/z 390.92 [M+H]^+ ion with a prominent product ion at m/z 229.00 [M+H]^+ (protonated resveratrol), which indicates the loss of the glucose moiety. Interestingly, the compound eluting at 24.7 min exhibited a nearly identical molecular weight (m/z 390.96) and CID spectrum (Fig. 4B(c)). In order to identify these compounds unambiguously, cis-piceid standards were generated using the UV-induced isomerization of the trans-piceid standards [3]. The peak at 24.7 min was confirmed as cis-piceid. Interestingly, a similar molecular weight (m/z 391.08) was recorded for the compound eluting at 16.1 min, and it also produced a UV spectrum (Fig. 4A(a)) identical to that of the trans-piceid peak. The CID spectrum of the m/z 391.08 produced product ions at m/z 229.00 and m/z 211.08, which indicated the sequential loss of glucose and hydroxyl units. The presence of m/z 211.08 [M+H]^+ indicated that the two hydroxyl groups were located in the A-ring of resveratrol (Fig. S1). These data strongly suggest that peak 1 at 16.1 min is not glucoside formed on the A-ring but a glucoside on the B-ring of resveratrol (resveratrol 4'-O-glucoside;

![Image](49x130 to 545x408)

**Fig. 4.** (A) UV and (B) MS spectra for the distinct peaks ((a) 16.1, (b) 20.1 and (c) 24.7 min) detected in the engineered *E. coli*. The dotted line in inset A(b) show the UV spectrum of the trans-piceid standard.
resveratrol oside). However, the resveratrol 4'-O-glucoside cannot be confirmed as having a trans- or cis- form. Furthermore, the fermentation products of the E. coli cells harboring pET-opTLS demonstrated that trans-resveratrol was a major product. Thus, the product of cis-piceid in the E. coli cells harboring pET-opTLS is likely to result from the glucosylation step may facilitate the isomerization for long incubations in the culture medium. The amount of the trans-piceid was detected as 2.5 mg/l at the end of cultivation via the quantification of the corresponding standard. On the other hand, the cis-piceid and the resveratrol 4'-O-glucoside were roughly identified as 1.7 and 7.5 mg/l, respectively, via comparison with the spectral data interpretation values of the trans-piceid.

In conclusion, an E. coli system that contains an artificial biosynthetic pathway that produces resveratrol 3-O-glucoside and resveratrol 4'-O-glucoside from simple carbon source cultures was developed.

Acknowledgments

This work was supported in part by a grant from the KRRIB Research Initiative Program and Basic Science Research program (2012-0001421) funded by the NRF and by the Next-Generation BioGreen 21 Program (SSAC, PJ00954022013) funded by the RDA, Republic of Korea.

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