De novo Biosynthesis of Tyrosol Acetate and Hydroxytyrosol Acetate from Glucose in Engineered *Escherichia Coli*

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Research

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Abstract

Background: Tyrosol and hydroxytyrosol derived from virgin olive oil and olives extract, have wide applications both as functional food components and as nutraceuticals. However, they have low bioavailability due to their low absorption and high metabolism in human liver and small intestine. Acetylation of tyrosol and hydroxytyrosol can effectively improve their bioavailability and thus increase their potential use in the food and cosmeceutical industries. There is no report on the bioproductin of tyrosol acetate and hydroxytyrosol acetate so far. Thus, it is of great significance to develop microbial cell factories for achieving tyrosol acetate or hydroxytyrosol acetate biosynthesis.

Results: In this study, two *de novo* biosynthetic pathways for the production of tyrosol acetate and hydroxytyrosol acetate were constructed in *Escherichia coli*. First, an engineered *E. coli* that allows production of tyrosol from simple carbon sources was established. Four aldehyde reductases were compared, and it was found that yeaE is the best aldehyde reductase for tyrosol accumulation. Subsequently, the pathway was extended for tyrosol acetate production by further overexpression of alcohol acetyltransferase ATF1 for the conversion of tyrosol to tyrosol acetate. Finally, the pathway was further extended for hydroxytyrosol acetate production by overexpression of 4-hydroxyphenylacetate 3-hydroxylase HpaBC.

Conclusion: We have successfully established the artificial biosynthetic pathway of tyrosol acetate and hydroxytyrosol acetate from fermentable sugars and demonstrated for the first time the direct fermentative production of tyrosol acetate and hydroxytyrosol acetate from glucose in engineered *E. coli*

Background

Plant phenolic compounds have antioxidant and other beneficial biological activities, so they have wide applications both as functional food components and as nutraceuticals [1]. Among these phenolic compounds, tyrosol and hydroxytyrosol derived from virgin olive oil and olives extract, have attracted extensive attention[2]. Tyrosol and hydroxytyrosol are widely recognized as an antioxidant, anti-inflammatory molecule that inhibits platelet aggregation and plays a protective role in the heart[3, 4]. However, they have low bioavailability due to their low absorption and high metabolism in human liver and small intestine[5].

Acetylation of phenolic compounds has been reported to enhance lipophilicity as well as absorption and cell permeability, thus improving their bioavailability[6]. Tyrosol acetate derivatives showed better antimicrobial and antileismaniac activities than tyrosol. This effect can be attributed to its increased lipophilicity[7, 8]. Tyrosol acetate also showed a higher cytotoxic effect on cancer lines than tyrosol[9]. On the other hand, hydroxytyrosol acylation can improve antioxidant activity, decrease tumor necrosis factor (TNF) and interleukin (IL) 1B plasma levels, decrease IL1B and chemokine ligand 2 levels of adipose tissue and showed greater anti-inflammatory effects than hydroxytyrosol[10–12]. This acetylation can be explored by reaction with acid chlorides or acid anhydrides, but these chemical routes do not meet the

requirements necessary for food applications. Several enzymatic methods have been reported for the preparation of tyrosol acetate or hydroxytyrosol acetate[13]. However, there are some drawbacks by using enzymes in bioprocesses such as the need of expensive enzymes and substrates. Thus, it is of great significance to develop microbial cell factories for achieving tyrosol acetate or hydroxytyrosol acetate biosynthesis. In this study, two *de novo* biosynthetic pathways for the production of tyrosol acetate and hydroxytyrosol acetate were constructed in *E. coli* (Fig. 1).

Methods

Plasmid and strains

Plasmid pDG11 for expression of the feedback resistant mutant of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase AroG^{fbr} (D146N) was constructed in our previous study[17]. The feedback resistant mutant of chorismate mutase/prephenate dehydrogenase tyrA^{fbr} was synthesized by Genewiz Biotech Co. Ltd., amplified by PCR using primers tyrA^{fbr}-XbaI and tyrA^{fbr}-SpeI-SalI. The XbaI-SalI fragment of tyrA^{fbr} was inserted into SpeI and SalI sites of pDG11 to give pDG20. The ketoacid decarboxylase KDC gene were amplified by PCR from from *S. cerevisiae* genomic DNA using primers KDC-XbaI and KDC-NheI-BamHI, and inserted into pBBRMCS1 via XbaI and BamHI to to give pDG21. The XbaI-SalI fragment of AroG^{fbr} and tyrA^{fbr} from pDG20 was inserted into NheI and XhoI sites of pDG21 to give pDG22.

Aldehyde reductases (gahK, dkgB, yeaE and gyhD) were individually amplified by PCR from *E. coli* genomic DNA using primers gahK – XbaI/gahK – NheI – BamHI, dkgB – XbaI/ dkgB – NheI –BamH, yeaE – XbaI/ yeaE – NheI – BamHI and gyhD–XbaI / gyhD – NheI – BamHI, and ligated into pET28a(+) via XbaI and BamHI to yield plasmid pDG23, pDG24, pDG25 and pDG26. Alcohol acetyltransferase ATF1 gene was amplified by PCR from *S. cerevisiae* genomic DNA using primers ATF1-XbaI and ATF1-NheI-SacI, and inserted into pET28a(+) to give pDG27. The XbaI-XhoI fragment of ATF1 from pDG27 was inserted into NheI and XhoI sites of pDG25 to give pDG28. 4-Hydroxyphenylacetate 3-hydroxylase HpaBC was amplified by PCR from *E. coli* genome using primers HpaBC-XbaI and HpaBC-XhoI, and inserted into NheI and XhoI sites of pDG28 to give pDG29. The strains, primers and plasmids used in this study are summarized in Table 1 and Table 2.

Table 1
Primers used in this study.

Primer name	Sequence (5'-3')
$tyrA^{fbr}$-XbaI	GTATCTAGAAAGAGGAGATATAATGGTTGCTGAATTGACCGCAT
$tyrA^{fbr}$-SpeI-SalI	ATATGTCGACACTAGTTTACTGGCGATTGTCATTCGCC
KDC-XbaI	atctctagatttaagaaggagatataatggcacctgttacaattgaaaagttc
KDC-NheI-BamHI	tctggatccgctagcctatttttttatttcttttaagtgccgctg
gahK – XbaI	ATCTCTAGATTTAAGAAGGAGATATAATGAAGATCAAAGCTGTTGGTGCATAT
gahK – NheI – BamHI	TCTGGATCCGCTAGCTCAGTCTGTTAGTGTGCGATTATCGATAA
dkgB – XbaI	ATCTCTAGATTTAAGAAGGAGATATAATGGCTATCCCTGCATTTGGTTTAG
dkgB – NheI – BamH	TCTGGATCCGCTAGCTTAATCCCATTCAGGAGCCAGACC
yeaE – XbaI	ATCTCTAGATTTAAGAAGGAGATATAATGCAACAAAAAATGATTCAATTTAGTGG
yeaE – NheI – BamH	TCTGGATCCGCTAGCTCACACCATATCCAGCGCAGTTT
gyhD–XbaI	ATCTCTAGATTTAAGAAGGAGATATAATGAACAACTTTAATCTGCACACCCC
gyhD – NheI – BamHI	TCTGGATCCGCTAGCTTAGCGGGCGGCTTCGTATATAC
ATF1-XbaI	GGATCTAGAAACTTTAAGAAGGAGATATAATGAATGAAATCGATGAGAAAAATCAGG
ATF1-NheI-SacI	GATGAGCTCACTAGTCTAAGGGCCTAAAAGGAGAGCTTTGTAA
HpaBC-XbaI	tagtctagatttaagaaggagatataatgaaaccagaagatttccgcg
HpaBC-XhoI	aacgctcgagttaaatcgcagcttccatttcca

Table 2
Plasmids and strains used in this study

Plasmids	Properties	Source
pDG22	pBBRMCS1; pT7: KDC, aroG ^{fbr} and tyrA^{fbr}	This study
pDG23	pET28a; pT7: gahK	This study
pDG24	pET28a; pT7: dkgB	This study
pDG25	pET28a; pT7: yeaE	This study
pDG26	pET28a; pT7: gyhD	This study
pDG28	pET28a; pT7: yeaE and ATF1	This study
pDG29	pET28a; pT7: yeaE, ATF1 and HpaBC	This study
Strains		
DG01	BL21 / pDG 22	This study
DG02	BL21 / pDG 22/ pDG23	This study
DG03	BL21 / pDG 22/ pDG24	This study
DG04	BL21 / pDG 22/ pDG25	This study
DG05	BL21 / pDG 22/ pDG26	This study
DG06	BL21 / pDG 22/ pDG28	This study
DG07	BL21 / pDG 22/ pDG29	This study

Shake Flask Cultures

Strains were cultured in 100 mL M9 medium with 2% glucose at 30°C as previously described by Guo[17]. 36 mg/L chloromycetin and 50 mg/L kanamycin were added to M9 medium. When the medium OD_{600} reached about 0.8, isopropyl β-D-thiogalactoside was added with a concentration of 0.1 mM.

Analytical Methods

Cultures samples (5 mL for tyrosol or 10 mL for tyrosol acetate/hydroxytyrosol acetate) were transferred to 20-mL glass tubes. Glass beads (0.1 mm) were used for breaking the cells by vigorous vortexing for 5 minutes. Equal volume of ethyl acetate was used to extract fermentation products. After the centrifuge stratification, the upper ethyl acetate phase was withdrawn, evaporated to near dryness, and redissolved in 1.0 mL of ethyl acetate. A 1 μL of the ethyl acetate phase was analysed after splitless injection on an Agilent 7890A GC equipped with an Agilent 5975 MS detector and an Agilent HP−5MS capillary column.

Helium was used as carrier gas. The temperatures of the injector and detector were 300°C and 250°C, respectively. The following temperature programme was applied: 80°C for 2 min, increase of 20°C min to 260°C, 260°C for 8 min. Benzyl alcohol was used as internal standard for quantitative tyrosol. Methyl benzoate was used as internal standard for quantitative tyrosol acetate and hydroxytyrosol acetate.

Result

Screening aldehyde reductase for biosynthesis of tyrosol

Recently, several research groups have reported the biosynthesis of tyrosol in *E. coli* using the intrinsic aldehyde reductase of *E. coli*[14–16]. However, which aldehyde reductase is primarily responsible for catalyzing the reduction of 4-hydroxyphenylacetaldehyde to tyrosol has not been identified. Aldehyde reductase is a superfamily enzyme which catalyze the reduction of a large variety of aldehydes. There are multiple aldehyde reductases in *E. coli*, such as gahK, yeaE, dkgB and gyhD. In this study, we assembled a tyrosol biosynthetic pathway with various aldehyde reductases in *E. coli* and assessed these aldehyde dehydrogenases for biosynthesis of tyrosol from glucose. The biosynthetic pathway consists of four enzymes: aroG^{fbr} and TyrA^{fbr} for the efficient overproduction of 4-hydroxyphenylpyruvic acid, ketoacid decarboxylase KDC from *S. cerevisiae* for the conversion of 4-hydroxyphenylpyruvic acid to 4-hydroxyphenylacetaldehyde, aldehyde reductase from *E. coli* for the conversion of 4-hydroxyphenylacetaldehyde to tyrosol. The resulting strains DG01-05 were grown in M9 medium with 20 g/L of glucose. The fermentation products were extracted by ethyl acetate and analyzed by GC–MS (Fig. 2). The tyrosol yield of the strains with overexpressing different aldehyde reductase were compared with that of the control strain without overexpressing aldehyde reductase. The results showed that yeaE was the most effective aldehyde reductase for biosynthesis of tyrosol, which resulted in an approximately 34% increase in tyrosol production up to 685 ± 31.1 mg/L compared the control strain DG01 (Table 3).

Table 3
Tyrosol production in engineered *E. coli* strains. All experiments were performed in triplicate and SD is indicated.

Strains	Tyrosol (mg/L)
DG01	512 ± 28.3
DG02	558 ± 21.2
DG03	614 ± 32.5
DG04	685 ± 31.1
DG05	631 ± 22.6

Besides tyrosol, the small amounts of phenylethanol, phenylethyl acetate and tyrosol acetate were also observed in these recombinant *E. coli* strains, as revealed by GC/MS analysis (Fig. 2). We infer that phenylpyruvate, L-phenylalanine pathway intermediate, can be decarboxylated by 2-keto acid decarboxylase KDC to phenylacetaldehyde, which is subsequently reduced by aldehyde dehydrogenase to phenylethanol. Subsequently, a small amount of phenylethanol and tyrosol were converted to phenylethyl acetate and tyrosol acetate by an intrinsic alcohol acetyltransferase-like enzyme of *E. coli*[17].

Production Tyrosol Acetate From Glucose

A small amount of tyrosol acetate was produced from tyrosol via an intrinsic alcohol acetyltransferase-like enzyme of *E. coli*. However, the efficiency is quite low. In order to improve the biosynthesis of tyrosol acetate, strain needs to strengthen the activity of alcohol acetyltransferase. Alcohol acetyltransferase ATF1 from *S. cerevisiae* has been used for acetylation of a variety of alcohol [18–21]. Therefore, we speculated that the observed promiscuity of the ATF1 can extend also to tyrosol. In this study, a fermentative route for biosynthesis of tyrosol acetate was created by further heterologous expression of ATF1 for the conversion of tyrosol to tyrosol acetate in the tyrosol-producing strain. The fermentation products were extracted by ethyl acetate and analyzed by GC–MS (Fig. 3). The resulting *E. coli* strain DG06 produced up to 507 ± 16.9 mg/L tyrosol acetate within 28 h (Table 4). This proves that the overexpression of ATF1 can effectively enhance the acetylation of tyrosol.

Table 4
Tyrosol acetate and hydroxytyrosol acetate production in engineered *E. coli* strains. All experiments were performed in triplicate and SD is indicated.

Strains	Tyrosol acetate(mg/L)	Hydroxytyrosol acetate(mg/L)
DG06	507 ± 16.9	
DG07	281 ± 11.3	225 ± 9.8

Production Hydroxytyrosol Acetate From Glucose

4-Hydroxyphenylacetate 3-hydroxylase HpaBC is widely distributed in many microorganisms including *E. coli*. HpaBC from *E. coli* can catalyze the hydroxylation of tyrosol to hydroxytyrosol[22]. Therefore, we hypothesized that the hydroxytyrosol acetate could be biosynthesized by tyrosol-producing *E. coli* through the expression of HpaBC that catalyzes the hydroxylation of tyrosol to hydroxytyrosol and alcohol acetyltransferase ATF1 that catalyzes the acetylation of hydroxytyrosol to hydroxytyrosol acetate. In this study, the pathway was further extended for hydroxytyrosol acetate production by further overexpression of HpaBC and ATF1 in the tyrosol-producing strain. The resulting *E. coli* strain DG07 produced up to 225 ± 9.8 mg/L hydroxytyrosol acetate within 28 h (Table 4). This proves that the designed hydroxytyrosol acetate synthetic pathway from glucose was effective in *E. coli*.

Discussion

Acetylated tyrosol and hydroxytyrosol can effectively improve their lipophilicity and thus promote their absorption and cell permeability[12, 23, 24]. This acetylation can be accomplished by acid-base catalysis, but these chemical methods do not meet the requirements necessary for food applications. In recent years, a number of pioneering studies have demonstrated the application of lipase for the bioproduction of tyrosol acetate and hydroxytyrosol acetate[13]. Aissa et al. reported that tyrosol acetate was synthesized using immobilized *Staphylococcus xylosus* lipase through transesterification between ethyl acetate and tyrosol[13]. Alcudia et al. reported that the synthesis of hydroxytyrosol acetate by pancreatic lipase with 86% yield after 48 h of reaction[25]. Although enzymes are preferred over chemical catalyst, it is not used widely in industry due to the high cost of enzymes and substrates.

To meet the increasing market demand, engineering microbes to produce tyrosol acetate and hydroxytyrosol acetate from renewable resources represents a promising alternative. In this study, two *de novo* biosynthetic pathways for the production of tyrosol acetate and hydroxytyrosol acetate were constructed in *E coli*. First, an engineered *E. coli* that allows production of tyrosol from simple carbon sources was established. Four aldehyde reductase were compared, and it was found that yeaE is the best aldehyde reductase for tyrosol accumulation. Subsequently, the pathway was further extended for tyrosol acetate and hydroxytyrosol acetate production by further overexpression of ATF1 and HpaBC. Although additional work is needed to reach commercial target levels, the present study opens up a new direction for engineering microbial production of tyrosol acetate and hydroxytyrosol acetate from cheap and readily-available renewable raw materials in the future.

Conclusion

Tyrosol acetate and hydroxytyrosol acetate have wide applications both as functional food components and as nutraceuticals. There is no report on the bioproductin of them so far. In this study, we demonstrated for the first time the development of microbial cell factories for achieving tyrosol acetate or hydroxytyrosol acetate biosynthesis.

Declarations

Availability of data and materials

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

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Authors' contributions

DG and HP conceived and designed the experiments. DG, XF and YS performed the experiments. DG, XF, YS and XL analyzed the data. DG and HP wrote the paper. All authors read and approved the manuscript.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

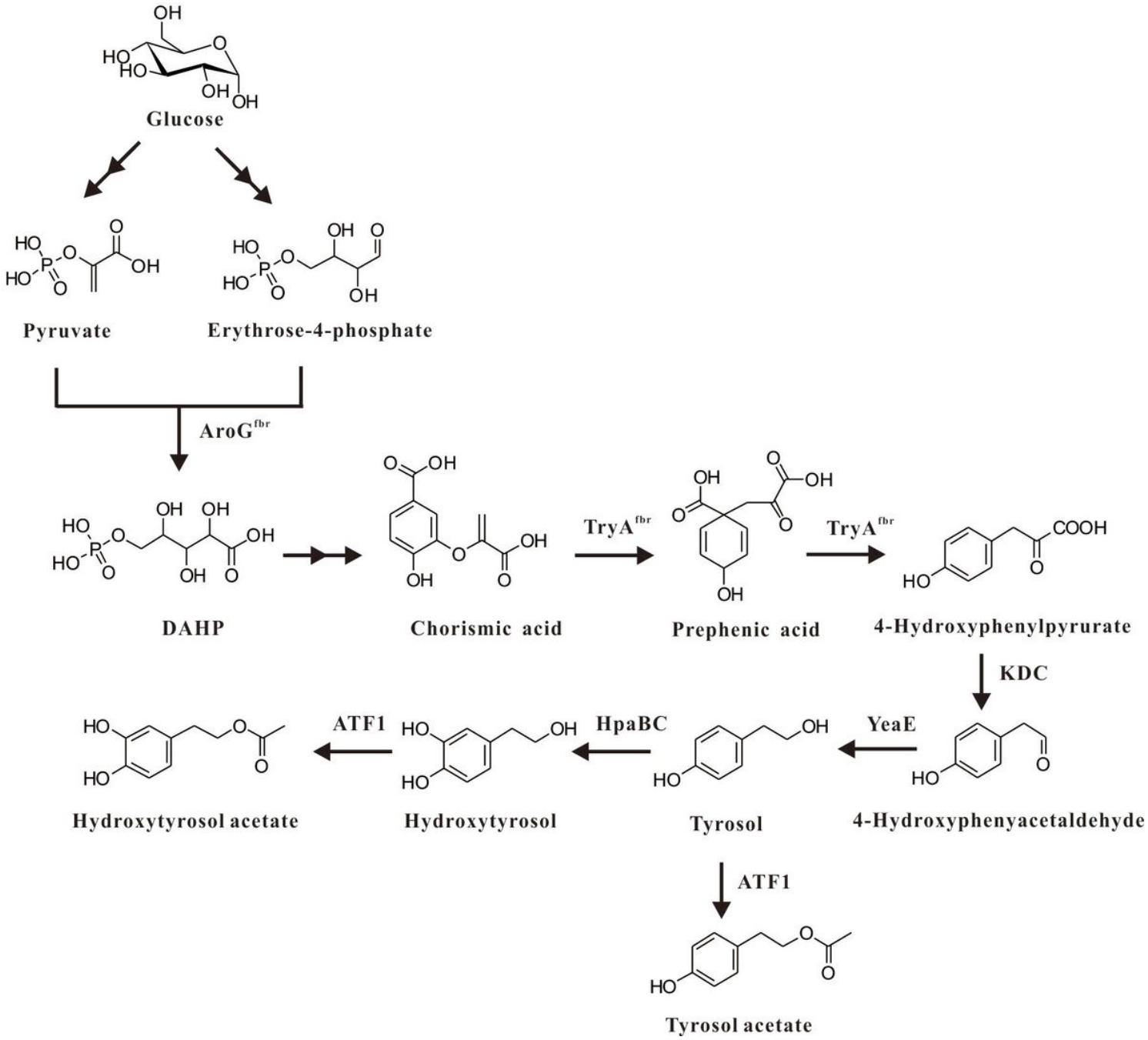

Figure 1

Engineered pathway for the production of tyrosol acetat and hydroxytyrosol acetate from glucose.

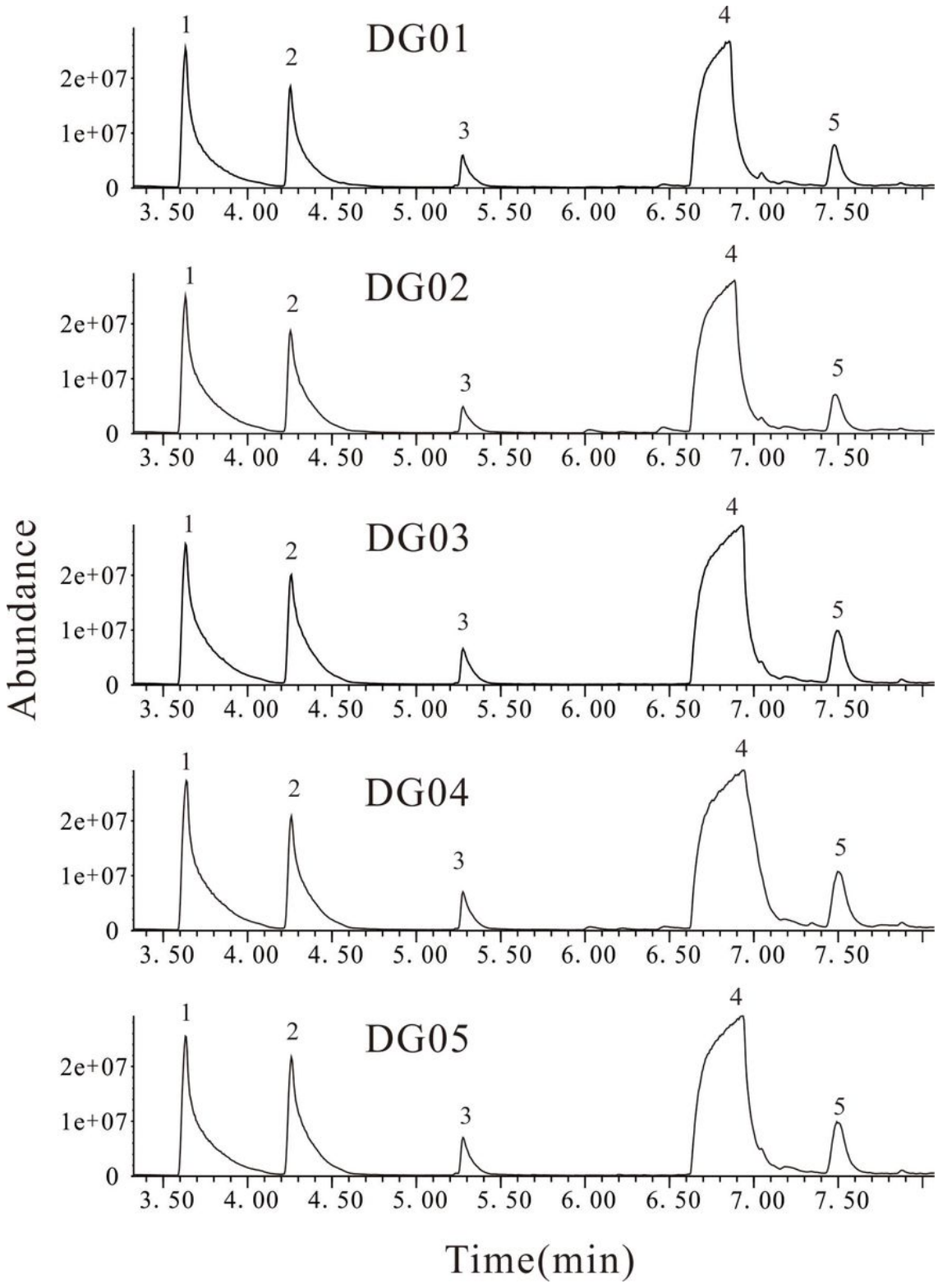

Figure 2

GC/MS analyses of tyrosol in engineered E. coli strains. Identified substances: 1, benzyl alcohol (internal standard); 2, phenylethanol; 3, phenylethyl acetate; 4, tyrosol; 5, tyrosol acetate.

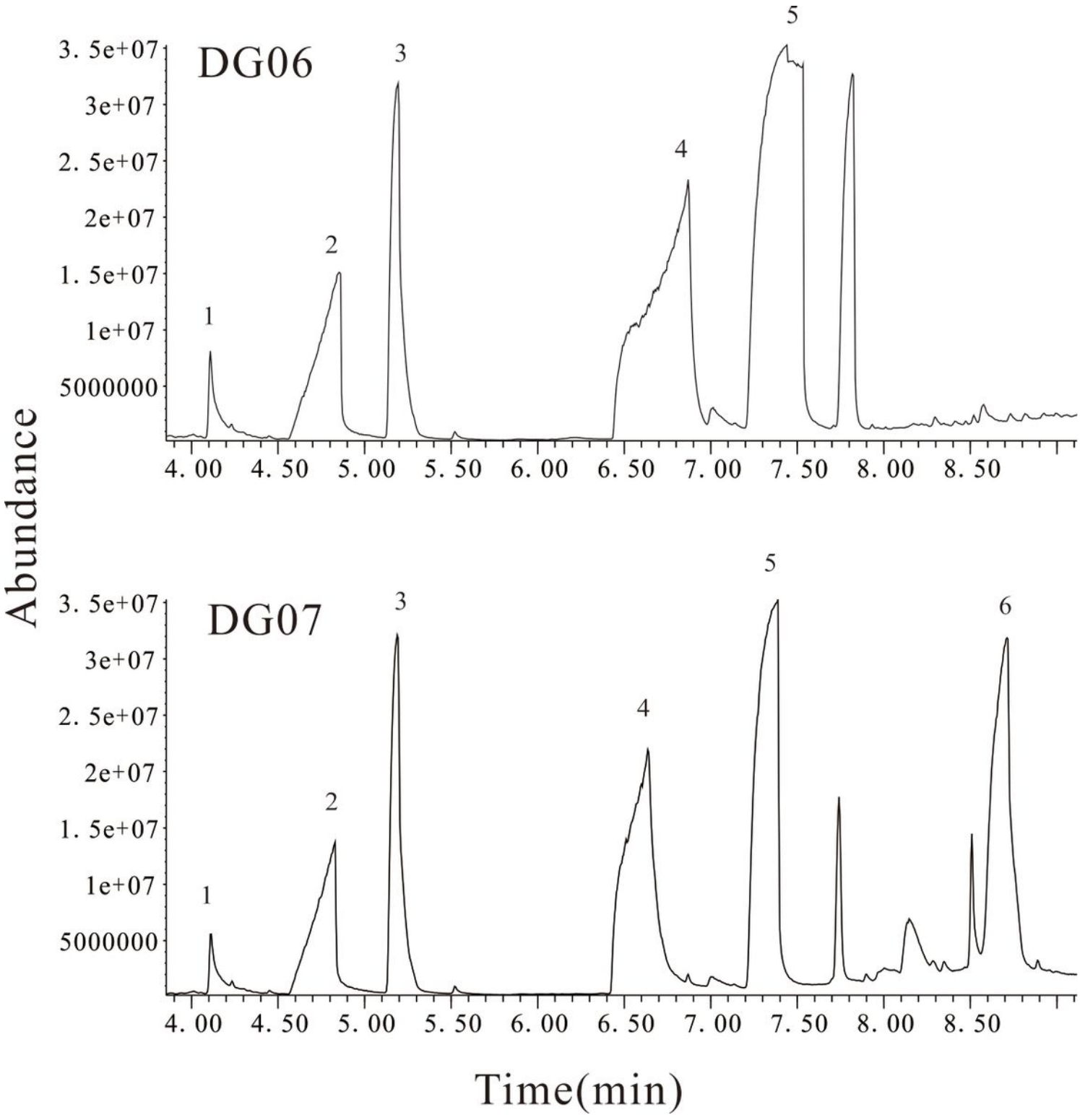

Figure 3

GC/MS analyses of tyrosol acetate and hydroxytyrosol acetate in engineered E. coli strains. Identified substances: 1, phenylethanol; 2, methyl benzoate (internal standard); 3, phenylethyl acetate; 4, tyrosol; 5, tyrosol acetate; 6, hydroxytyrosol acetate.