Increased Carvone Production in Escherichia Coli by Balancing Expression of Limonene Conversion Enzymes Through Targeted QconCAT Proteome Analysis

Erika Yoshida (erika_yoshida@ajinomoto.com)
Ajinomoto Co., Inc. https://orcid.org/0000-0003-2201-8108
Motoki Kojima
KNC Laboratories Co., Ltd.
Munenori Suzuki
KNC Laboratories Co., Ltd.
Fumio Matsuda
Osaka University: Osaka Daigaku
Akiko Onuki
Ajinomoto Co., Inc.
Yousuke Nishio
Ajinomoto Co., Inc.
Yoshihiro Usuda
Ajinomoto Co., Inc.
Akihiko Kondo
Kobe University: Kobe Daigaku
Jun Ishii
Kobe University: Kobe Daigaku

Research

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Abstract

Background

Natural mint flavor is produced by extraction from mint, which is not efficient enough to make it environment friendly process. (−)-Carvone is the monoterpenoid with key flavor of spearmint, and there has been an attempt to produce (−)-carvone by recombinant *Escherichia coli.* Although all enzymes in (−)-carvone biosynthesis have been functionally expressed in *E. coli* independently, the yield of (−)-carvone was low in the previous study.

Results

We have found a by-product formation when cytochrome P450 limonene-6-hydroxylase (P450)/cytochrome P450 reductase (CPR) and carveol dehydrogenase (CDH) were expressed in single cell. These by-products were determined as dihydrocarveol and dihydrocarvone. We hypothesized that the enzymatic kinetics and the expression levels of P450 and CDH are quite different in *E. coli*. Therefore, two strains independently expressing P450/CPR and CDH were mixed with different mixing ratio, confirming increase in carvone production and decrease in by-product formation when CDH input was reduced. To determine the optimum balance of enzyme expressions, proteome analysis quantification concatemer (QconCAT) method to quantify P450, CPR, and CDH was developed. Using the QconCAT standard protein that was artificially created by concatenating the tryptic peptides, the ratio between P450 and CDH was calculated, and their optimum ratio to maximize (−)-carvone production was shown. Then, a single strain expressing both P450/CPR and CDH was constructed to imitate the superior expression ratio. The upgraded strain showed 15-fold improvement compared to the initial strain, showing 44 ± 6.3 mg/L of (−)-carvone production from 100 mg/L (−)-limonene as a starting substrate.

Conclusions

Improved expression balance of P450 and CDH in recombinant *E. coli* increased the (−)-carvone production using (−)-limonene as the direct substrates by the whole-cell biocatalysis, showing approximately 150 times higher titer than previous report. Our study showed the usefulness of proteome analysis QconCAT method in the strain development for industrial biotechnology field.

Background

(−)-Carvone is a member of monoterpenoid and key flavor compound of spearmint essential oil [1]. (−)-Carvone is utilized for spearmint flavor and fragrance in segments such as confectionery and oral care [2]. The annual production of (−)-carvone is approximately 3,800 tons per year, the majority of which (approximately 2,000 tons per year) is made by chemical synthesis from (+)-limonene [3]. Due to enhanced health- and environment-awareness in recent years, more consumers prefer natural flavors and fragrances for better perception [4]. Spearmint flavor is not an exception, so that the demand for natural spearmint flavor, or natural (−)-carvone is increasing. The spearmint essential oil is currently the only

source of natural spearmint flavor, including natural (−)-carvone. There has been an issue that the demand for natural spearmint flavor exceeds the supply. To increase the supply is not simple, since current spearmint essential oil production method is water-intensive and requires improvement to be sustainable. Also, spearmint cultivation can be easily affected by the weather, such as drought, so that the spearmint essential oil supply volume and unit price is fluctuating [5]. Therefore, it is desirable to develop sustainable and stable natural (−)-carvone production method in order to accommodate the market demands.

One possible solution is (−)-carvone production by fermentation in a microbe. There are numerous attempts to produce flavor and fragrance compounds by biotechnology rather than extracting from its natural sources, because it has potential to be more sustainable and stable [6]. The regulatory circumstance is also supporting such attempts. For example, the flavor and fragrance compounds produced by biotechnology (regardless of the microbial or enzymatic process) can be labeled as "natural," according to European regulation CE 1334/2008 [7]. Therefore, (−)-carvone produced by biotechnology method can be labeled as natural (−)-carvone, and sustainably replace natural (−)-carvone conventionally produced by extracting from spearmint. To our knowledge, there is no natural (−)-carvone produced by biotechnology in the market yet.

The purpose of our study is to develop the sustainable and cost-effective replacement of carvone production by using microbes. (−)-Carvone is synthesized from the precursor (−)-limonene in its native producer spearmint. Specifically, intracellular (−)-limonene is converted to (−)-*trans*-carveol by cytochrome P450 limonene-6-hydroxylase (along with cytochrome P450 reductase; CPR), while (−)-*trans*-carveol is converted to (−)-carvone by carveol dehydrogenase (CDH) (Fig. 1). The enzymology of (−)-carvone biosynthesis in spearmint has been studied in detail [8,9]. The cytochrome P450 limonene-6-hydroxylase and CDH have been functionally expressed in *Escherichia coli* [10,11]. However, when these three genes were expressed in a single *E. coli* cell, a very low level of (−)-carvone (up to 2 μM) can be obtained from whole-cell biocatalysis with (−)-limonene supplementation [12]. The reason for the low conversion rate was still not clear, however, one general issue among heterologous expression of cytochrome P450 of plant origin is the difficulty to express in a heterologous host such as *E. coli* [13]. In order to increase the target compound production by plant origin cytochrome P450 in *E. coli*, careful fine-tuning at protein level to balance the expression of P450 along with other pathway enzymes was necessary [14]. Indeed, heterologous expression of cytochrome P450 limonene-6-hydroxylase required intensive N-terminal modification [10], whereas CDH was expressed well in soluble form without any modification [11]. Kinetic parameter information of these two enzymes were limited, but it appeared that P450 reaction was rate-limiting in spearmint plant [9]. Based on these prior studies, we hypothesized that the expression levels and the enzymatic kinetics of P450 and CDH are quite different, leading imbalance in the carvone biosynthesis pathway in *E. coli*, and ultimately causing the low conversion rate from (−)-limonene to (−)-carvone. To investigate such hypothesis, it was desired to have a protein quantification method with high sensitivity to conduct comparative study among various strains by abundance ratio of pathway enzymes.

Proteome analysis is a powerful tool for quantification of proteins. Proteome analysis can be divided into two types, relative quantification and absolute quantification. The relative quantification method in proteome analysis can be conducted without the laborious preparation of standard proteins; however, different proteins cannot be compared for expression level. The absolute quantification method requires a synthetic, isotope-labeled standard peptide (AQUA) preparation [15]. AQUA peptide preparation is still relatively expensive so that it is hardly performed in strain construction for industrial biotechnology fields. Another method for quantification standard preparation is by individually purify each target proteins in an isotope-labeled form, called the PSAQ method [16]. Alternatively, the quantification concatemer (QconCAT) method enables the standard peptide preparation easier and cheaper [17]. In the QconCAT method, targeted peptides are concatenated into a QconCAT protein, which is spiked in as a standard in proteome analysis. For our purpose, QconCAT method appears to be an attractive choice. There are a few studies that used the QconCAT method for quantification of proteins in prokaryote [18–20]; however, to our knowledge, there is no reports that the QconCAT method was applied to genetic engineering for upgrading metabolic pathways. In recent years, DNA synthesis became extremely accessible, so that the QconCAT method has a potential to become more popular among the synthetic biology field.

In this study, we developed a QconCAT method to quantify the expression of carvone biosynthetic pathway enzymes, P450, CPR and CDH. Upon receiving our hypothesis of imbalanced P450 and CDH expression in *E. coli*, two strains independently expressing P450/CPR and CDH were mixed with different mixing ratio, and the QconCAT method was used to determine the optimum expression balance between the P450 and CDH. Based on this proteome analysis data, a single strain expressing both P450/CPR and CDH with superior expression ratio was constructed. This upgraded strain displayed 15-fold improvement of (−)-carvone production compared to our initial strain, and achieved increase of (−)-carvone titer approximately 150 times higher than the previous report. To our knowledge, this is the first report in genetic engineering for upgrading metabolic pathways using the QconCAT method.

Results

Cloning and functional expression of P450 genes in E. coli

The cytochrome P450 (−)-(4*S*)-limonene-6-hydroxylase gene *CYP71D18* of spearmint (*Mentha spicata*) was codon-optimized for *E. coli*, and cytochrome P450 reductase (CPR) gene *ATR2* of *Arabidopsis thaliana* was used as the native sequence. These two genes were cloned into pCDFDuet-1 vector and were validated to be consistent with the designed sequences. *E. coli* BL21(DE3) transformants harboring pCDF-ATR2 (named as Ma strain) or pCDF-CYP71D18-ATR2 (named as Mpa strain) were induced with IPTG, and whole-cell P450 enzymatic activity was tested with supplementation of (−)-limonene as a substrate. *E. coli* strain Mpa, which is expressing both P450 and CPR demonstrated conversion of (−)-limonene to (−)-*trans*-carveol (Fig. 2A). Whole cell activity was defined as final concentration of (−)-*trans*-carveol per hour per OD_{600} ($mg \times L^{-1}\ h^{-1}\ OD_{600}{}^{-1}$). Mpa cells showed whole cell P450 activity of 2.4 ($mg \times L^{-1}\ h^{-1}\ OD_{600}{}^{-1}$).

Cloning and functional expression of CDH genes in E. coli

For carveol dehydrogenase (CDH), we have tested two genes, one from peppermint (*Mentha x piperita*) and another one from *Rhodococcus erythropolis*. First, CDH gene *ISPD* of peppermint was codon-optimized and cloned into pET-3a vector. The sequence was validated to be consistent with the designed sequence. *E. coli* BL21(DE3) transformant harboring pET-ISPD (named as Hc strain) was induced with IPTG, and whole-cell CDH enzymatic activity was tested with supplementation of (−)-carveol as a substrate (commercially available (−)-carveol contains (−)-*trans*-carveol and (−)-*cis*-carveol, and trace of (−)-carvone). *E. coli* strain Hc, which is expressing ISPD, demonstrated conversion of (−)-*trans*-carveol to (−)-carvone (Fig. 2B). The CDH activity of ISPD-expressing cells was specific towards (−)-*trans*-carveol and not to (−)-*cis*-carveol, which is consistent with a previous study [11]. Whole cell CDH activity was defined as final concentration (−)-carvone of per hour per OD_{600} ($mg \times L^{-1}\ h^{-1}\ OD_{600}{}^{-1}$). Hc cells showed whole cell CDH activity of 4.9 ($mg \times L^{-1}\ h^{-1}\ OD_{600}{}^{-1}$).

In addition to CDH from peppermint (ISPD), another CDH gene *limC* from *R. erythropolis* DCL14 was also codon-optimized and cloned into pET-3a and expressed in *E. coli*. There was no previous report of heterologous expression of this gene. *E. coli* BL21(DE3) transformant harboring pET-limC was induced with IPTG at various conditions, and SDS-PAGE of the cell lysate revealed a strong band with the molecular mass of 30 kDa, which corresponds to the size of CDH from *R. erythropolis* DCL14 (Additional file 1. A). This result indicated that CDH gene *limC* was successfully expressed in *E. coli* BL21(DE3). Next, CDH enzymatic activity of expressed LimC was tested with supplementation of (−)-carveol as a substrate. *E. coli* strain expressing *limC* gene demonstrated conversion of (−)-carveol to (−)-carvone only under the presence of artificial electron acceptor dichlorophenolindophenol (DCPIP), as described in the previous study [21] (Additional file 1. B). Additional supplementation of artificial electron acceptor is not desirable in industrial production; therefore, we have selected CDH gene *ISPD* from peppermint in the remaining study.

Biocatalysis of (−)-carvone from (−)-limonene

The cytochrome P450 limonene 6-hydroxylase gene *CYP71D18* of spearmint (referred to as P450 thereafter), cytochrome P450 reductase gene *ATR2* of *A. thaliana* (referred to as CPR thereafter) and carveol dehydrogenase gene ISPD of peppermint (referred to as CDH thereafter) were individually confirmed its functional expression in *E. coli*. As a next step, a strain co-expressing P450, CPR and CDH was constructed (BL21(DE3) pCDF-CYP71D18-ATR2-ISPD, named as Mpac strain) and incubated with (−)-limonene as a substrate at different two temperatures. As a result, (−)-carvone was detected along with undesired by-product formation (Fig. 3A). Out of two reaction temperature conditions tested, 14 °C condition showed higher production of (−)-carvone than 20 °C condition. To identify the cause of these by-product formations, P450, CPR, and CDH co-expressing strain (Mpac) and P450 and CPR expressing strain (Mpa) were incubated with (−)-carveol as a substrate, and different pattern of by-product formation was observed (Fig. 3B). Specifically, Mpac strain expressing CDH generated compound #5, while Mpa strain that did not express CDH generated compound #6 with a small quantity of compound #7 (Fig. 3B).

Similarity search of mass spectrometry (MS) fragment pattern, and further analysis of conversion mixture and authentic compound suggested that these by-products were dihydrocarveol (#5) and dihydrocarvone (#6) (Fig. 3C). The minor peak, #7, was dihydrocarvone isomer (Fig. 3C). In addition, when (−)-carveol and (−)-carvone was incubated with wild type *E. coli* BL21(DE3) strain, dihydrocarvone formation was also observed (Additional file 2). These results indicated that the exogenous enzyme peppermint CDH (ISPD) generated dihydrocarveol and the endogenous *E. coli* enzyme generated dihydrocarvone as by-products.

Optimization of carvone biocatalysis by QconCAT proteome analysis

Since CDH was shown to cause the by-product formation, optimization of the expression ratio between P450/CPR and CDH was attempted. This experiment was conducted under the hypothesis that excessive CDH in the reaction may cause undesired by-product formation since whole-cell CDH activity was higher than whole-cell P450 activity. Mpa strain (BL21(DE3) harboring pCDF-CYP71D18-ATR2) and Hc strain (BL21(DE3) harboring pET-ISPD) were separately cultured, induced with IPTG, and mixed at the various ratio with substrate (−)-limonene. Results of Mpa and Hc strains mixed at 100:100, 100:10, 100:1 ratio respectively, based on the OD value, were shown in Fig. 4. When P450/CPR and CDH expressing strains were mixed at 100:1 ratio, the (−)-carvone concentration reached to the maximum, while dihydrocarvone peak was minimum. To corroborate the hypothesis, BL21(DE3) transformant harboring the high-copy CDH expression plasmid (pET-ISPD) along with pCDF-CYP71D18-ATR2 was generated (MpaHc strain) and confirmed that MpaHc presumably with high CDH expression drastically decreased the carvone production (Table 3).

Next, we aimed to determine the optimum ratio between P450/CPR and CDH at the protein level. One issue for such a strategy was that there was no method to quantify protein, especially P450. Heterologous expression of CYP71D18 was not detectable by SDS-PAGE analysis, while other proteins, CPR and CDH were readably detectable (data not shown). As data in Fig. 4 clearly showed that reducing CDH amount in the reaction is beneficial to increase (−)-carvone conversion, it is necessary to have the quantitative result of protein expression level to determine the optimum abundance ratio among pathway enzymes, so that the optimum ratio can be represented in the optimized strain. Therefore, the semi-quantitative proteome analysis method using quantification concatemer (QconCAT) protein was developed for P450, CPR, and CDH quantification.

In this method, we firstly determined the candidate peptides to be analyzed, based on the amino acid sequence of P450, CPR, and CDH. Next, we analyzed actual samples from strains expressing these proteins and selected two peptides for each protein by its detection strength. Then, artificial standard QconCAT proteins were constructed. In QconCAT protein design, each peptide to be analyzed in LC-MS/MS were concatenated next to each other. Two different designs by arranging different order were attempted, namely QconCAT1 and QconCAT2 (Fig. 5A). The DNA sequences corresponding to these artificial proteins were synthesized and inserted to pET-28a vector. *E. coli* BL21(DE3) transformants

harboring pET28a-QconCAT1 or pET28a-QconCAT2 were induced with IPTG, and SDS-PAGE of the cell lysate revealed a band with a molecular mass of 20 kDa, which corresponds to QconCAT protein as designed. Both were His-tag purified to single band purity (Fig. 5B).

These QconCAT proteins were treated with trypsin, and the detection of all peptide included in the design was confirmed in advance. We determined to use the QconCAT1 protein, of which the yield after purification was higher than the QconCAT2 (Fig. 5B), for further experiments. Then, the strain expressing QconCAT1 protein was cultured in the labeled medium where all glucose was substituted with [U-$^{13}C_6$] glucose, induced with IPTG, then purified as previously. This labeled purified QconCAT1 protein was used as a standard for proteome analysis. The proteome analysis was conducted with strains expressing P450, CPR, and CDH (MpaHc), P450 and CPR (Mpa) or CDH (Hc), and relative expression amount versus QconCAT1 was determined (Fig. 5C). It was clearly shown that in MpaHc strain, P450 expression was very low, whereas both CPR and CDH expressions were high.

Using the QconCAT proteome analysis result, the P450/CDH ratio corresponding to Mpa and Hc mixture (100:100, 100:10, 100:1 ratio respectfully, based on the OD value) and MpaHc was calculated, and its correlation between carvone production was shown (Fig. 6). In the tested condition, (−)-carvone concentration reached the maximum when the P450/CDH ratio was high as 16 (Mpa:Hc = 100:1).

Carvone conversion by single cell reaction

Based on the result of the QconCAT proteome analysis, the additional strain was constructed in order to represent improved balance of P450 and CDH expression in single cell. Since the CDH gene was previously expressed from a high copy vector (pET-3a), low copy vector pMW218 was selected as a new vector backbone to express CDH at decreased expression level. Constructed plasmid pMW-ISPD was introduced into the *E. coli* BL21(DE3) strain with P450 expression plasmid pCDF-CYP71D18-ATR2-ISPD (MpaLc). The novel strain MpaLc along with previously constructed strains with pET-ISPD (MpaHc) were analyzed using QconCAT proteome analysis and carvone biocatalysis assay. MpaLc produced 44 ± 6.3 mg/L of (−)-carvone from 100 mg/L (−)-limonene as a starting substrate, whereas MpaHc produced 2.9 ± 0.79 mg/L of (−)-carvone (Fig. 7). The ratio P450/CDH of MpaLc was 12 ± 1.5, whereas MpaHc was 0.004 ± 0.0008. The ratio P450/CDH was higher in MpaLc as designed, and carvone biocatalysis was increased as aimed.

Discussion

Proteome data is becoming more important and popular in metabolic engineering [22]. In our study, we have successfully demonstrated the benefit of targeted QconCAT proteome analysis in genetic engineering for upgrading metabolic pathways. The QconCAT method enables semi-quantitative analysis so that the abundance ratio between pathway enzymes can be determined. Such information is powerful when the imbalance of enzyme expression is a bottleneck in production strain. The relative quantification method in proteome analysis only allows comparison among the same protein in different strains, where

the semi-quantitative method in proteome analysis allows comparison among different proteins in different strains. In (−)-limonene production pathway, it was necessary to balance the expression level between P450 and CDH, since excessive CDH was shown to cause by-product formation. In order to investigate the optimum conversion condition, two strains independently expressing P450/CPR and CDH were mixed with different mixing ratio, then the QconCAT method revealed the optimum abundance ratio between P450/CPR and CDH.

Using this proteome data, upgraded strain was constructed to represent the optimum abundance ratio between the P450/CPR and CDH within a single cell. This upgraded strain displayed 15-fold improvement of (−)-carvone production compared to our initial strain and achieved increase of (−)-carvone titer approximately 150 times higher than the previous report [12]. To our knowledge, this is the first report in genetic engineering for upgrading metabolic pathways using the QconCAT method. Imbalance of pathway enzyme expression is a very common issue in genetic engineering, and we believe that semi-quantitative proteome analysis would provide clear answer in strain development strategy. The QconCAT method requires the preparation of labeled and purified QconCAT protein, but once it is prepared multiple rounds of the experiment can be performed. The downside of QconCAT is that the target protein to be measured cannot be changed after QconCAT preparation. It is required to decide the pathway design before constructing the QconCAT protein. Therefore, the QconCAT method is most effective at the later phase of the strain construction when the fine-tuning of pathway enzyme expression balance is required, rather than the earlier phase of the strain construction when pathway enzyme selection is being performed. The methodology we have developed can be applied in a wide variety of target compound in synthetic biology.

Our study shows for the first time, to our knowledge, that (−)-carvone biocatalysis from (−)-limonene in engineered *E. coli* can lead to the undesired by-product formation. Dihydrocarveol was detected when (−)-carveol was incubated with cells expressing CDH. Dihydrocarvone was detected when (−)-carveol was incubated with cells not expressing CDH. Dihydrocarvone was also produced when (−)-carvone was incubated with *E. coli* wild type strain. Based on these results, we propose a hypothetical by-product formation pathway (Fig. 8). ISPD, the CDH from peppermint, is reported to be active to the substrates such as (−)-*trans*-carveol, (−)-*trans*-isopiperitenol, (+)-neomenthol, and (+)-neoisomenthol [11]. ISPD is reported to be not active to substrates such as (−)-*cis*-carveol, (−)-menthol, (+)-isomenthol, and (−)-perillyl alcohol [11]. ISPD was unable to catalyze the reduction of (−)-isopiperitenone, nor (−)-carvone [11]. There was no data regarding ISPD's activity to dihydrocarveol or dihydrocarvone. The essential oil of spearmint is consists of carvone (51.7%), dihydrocarveol (11.5%), and *cis*-dihydrocarvone (9.1%) [1]. However, the enzyme which is responsible for the formation of dihydrocarveol and dihydrocarvone in spearmint is unknown. To elucidate this pathway, *in vitro* assay of ISPD is desired in future work. For the industrial purposes, it is strongly desired to reduce by-product formation so the overall production cost can be minimized. The *E. coli* endogenous gene which is responsible for the reaction from (−)-carvone to dihydrocarvone is also unknown. It is possible to search for the gene by screening, however, there could be multiple genes responsible for such reaction so that it requires multiple gene deletions. We took a rather realistic approach to overcome this situation, by reducing the CDH expression level to balance

against P450 and optimizing reaction condition to prevent further production of the by-products. In our final strain (MpaLc), dihydrocarveol and dihydrocarvone were still present. To reduce these by-products, further optimization is necessary.

The purpose of this study was to develop the production method of natural (−)-carvone by biotechnology. We have shown that *E. coli* whole cell bioconversion method from (−)-limonene as a feeding substrate. (−)-Limonene is a highly volatile compound and solubility to water is low. The uptake of the substrate can be the limitation of this reaction as prior study suggests [12]. Therefore, our next target is to integrate the developed pathway of carvone conversion with limonene biosynthesis pathway. Limonene producer strain has been reported in several literature [23,24]. We aim to construct limonene high producer strain then introduce carvone conversion pathway. We can take an approach to produce carvone by direct fermentation from substrates such as simple sugar. By doing so, intracellular limonene can be efficiently converted into carveol and carvone accordingly. Direct fermentation method with high titer and productivity can reduce production cost, and potentially becoming environment-friendly and cost-effective method of natural mint flavor production in near future.

Conclusions

The (−)-carvone biosynthesis pathway genes, cytochrome P450 limonene-6-hydroxylase, cytochrome P450 reductase, and carveol dehydrogenase from plants were introduced into *E. coli* to construct the whole-cell biocatalytic system. The whole-cell biocatalysis from (−)-limonene to (−)-carvone was attempted, and by-products such as dihydrocarveol and dihydrocarvone were detected in the reaction mixture. Best protein expression balance was determined using proteome analysis QconCAT method and resulted in strain representing the superior balance between cytochrome P450 limonene-6-hydroxylase and carveol dehydrogenase showed 15-fold improvement compared to the strain before engineering. Upgraded strain (MpaLc) produced 44 ± 6.3 mg/L of (−)-carvone from 100 mg/L (−)-limonene as a starting substrate, where control strain (MpaHc) produced 2.9 ± 0.79 mg/L of (−)-carvone and attained 150 times higher production titer than the previous report. Our study showed the usefulness of the proteome analysis QconCAT method in the industrial biotechnology field.

Methods

Plasmid construction

Plasmids used in this study were listed in Table 1. Codon-optimization of *CYP71D18* gene was done for *E. coli* using the GeneArt Strings™ DNA Fragments service by Life Technologies Corporation (Carlsbad, CA, USA). Codon-optimization of the remaining genes except for *ATR2* gene was done for *E. coli* using the OptimumGene algorithm by GenScript Biotech Corporation (Piscataway, NJ, USA). Detailed sequences are shown in additional file 3. Cytochrome P450 reductase (*ATR2*, Accession number NM_119167) gene derived from *Arabidopsis thaliana* was truncated by 72 amino acids to remove hypothetical membrane anchoring region and then tag sequence for soluble expression was added and inserted at multiple

cloning site 2 (MCS2) of pCDFDuet-1 vector to construct pCDF-ATR2 plasmid. Then, cytochrome P450 (*CYP71D18*, Accession number AF124815) gene derived from *Mentha spicata* was codon optimized for *E. coli* expression and truncated by 5 amino acids to remove hypothetical membrane anchoring region then tag sequence for soluble expression was added and inserted at multiple cloning site 1 (MCS1) of pCDF-ATR2 vector to construct pCDF-CYP71D18-ATR2. Carveol dehydrogenase (*ISPD*, Accession number AY641428) gene from *Mentha x piperita* was codon optimized for *E. coli* expression and inserted at SalI site (in between P450 and ATR2 genes) of pCDF-CYP71D18-ATR2 plasmid with SD sequence to construct pCDF-CYP71D18-ATR2-ISPD plasmid. *ISPD* gene was also inserted at NdeI-BamHI site of pET-3a plasmid to construct pET-ISPD plasmid. Similarly, *ISPD* was inserted at KpnI-SalI site of pMW218 to construct pMW-ISPD. Carveol dehydrogenase (*limC*, Accession number AJ006869) gene from *Rhodococcus erythropolis* DCL14 was codon optimized for *E. coli* expression and inserted at NdeI-BamHI site of pET-3a plasmid to construct pET-limC plasmid. QconCAT1 gene (design detail is described in QconCAT standard protein preparation) was codon optimized for *E. coli* expression and inserted at BamHI-XhoI site of pET-28a plasmid to construct pET-QconCAT1 plasmid. QconCAT2 gene (design detail is described in QconCAT standard protein preparation) was codon optimized for *E. coli* expression and inserted at BamHI-XhoI site of pET-28a plasmid to construct pET-QconCAT2 plasmid.

Table 1
Plasmids used in this study

Plasmid name	Description	Source of reference
pCDFDuet-1	Expression vector carrying two multiple cloning sites with T7*lac* promoter and ribosome biding site, CloDF13-derived CDF replicon, *lacI* gene and streptomycin/spectinomycin resistance gene.	Novagen
pCDF-ATR2	Cytochrome P450 reductase (*ATR2*, Accession number NM_119167) gene derived from *Arabidopsis thaliana* was inserted at multiple cloning site 2 (MCS2) of pCDFDuet-1.	This study
pCDF-CYP71D18-ATR2	Cytochrome P450 (*CYP71D18*, Accession number AF124815) gene derived from *Mentha spicata* was inserted at multiple cloning site 1 (MCS1) of pCDF-ATR2.	This study
pCDF-CYP71D18-ATR2-ISPD	Carveol dehydrogenase (*ISPD*, Accession number AY641428) gene from *Mentha x piperita* was inserted at SalI site (in between P450 and CPR genes) of pCDF-CYP71D18-ATR2	This study
pET-3a	Expression vector carrying one cloning site with T7 promoter and ribosome biding site, pBR322 replicon, and ampicillin resistance gene.	Novagen
pET-ISPD	Carveol dehydrogenase (*ISPD*, Accession number AY641428) gene from *Mentha x piperita* was inserted at NdeI-BamHI site of pET-3a.	This study
pMW218	Cloning vector carrying one multiple cloning site, pSC101 replicon, and kanamycin resistance gene.	Nippon Gene
pMW-ISPD	Carveol dehydrogenase (*ISPD*, Accession number AY641428) gene from *Mentha x piperita* was inserted at KpnI-SalI site of pMW218.	This study
pET-limC	Carveol dehydrogenase (*limC*, Accession number AJ006869) gene from *Rhodococcus erythropolis* DCL14 was inserted at NdeI-BamHI site of pET-3a.	This study
pET-28a	Expression vector carrying one cloning site with T7*lac* promoter and ribosome biding site, N-terminal His•Tag®/thrombin/T7•Tag® configuration, pBR322 replicon, and kanamycin resistance gene.	Novagen
pET-QconCAT1	QconCAT1 sequence was inserted at BamHI-XhoI site of pET-28a.	This study
pET-QconCAT2	QconCAT2 sequence was inserted at BamHI-XhoI site of pET-28a.	This study

Strains

Strains used in this study were listed in Table 2. *E. coli* BL21(DE3) was used as the host strain for protein expression and biocatalysis.

Table 2
Strains used in this study

Strain	Parental strain	Plasmid
Ma	BL21(DE3)	pCDF-ATR2
Mpa	BL21(DE3)	pCDF-CYP71D18-ATR2
Mpac	BL21(DE3)	pCDF-CYP71D18-ATR2-ISPD
Hc	BL21(DE3)	pET-ISPD
H	BL21(DE3)	pET
MpaHc	BL21(DE3)	pCDF-CYP71D18-ATR2, pET-ISPD
Lc	BL21(DE3)	pMW-ISPD
MpaLc	BL21(DE3)	pCDF-CYP71D18-ATR2, pMW-ISPD

Culture method and biocatalysis condition

The recombinant strain BL21(DE3) harboring expression plasmids were grown at 37 °C in LB medium as the seed culture. The seed culture was inoculated into 20 mL of TB broth medium (Bacto tryptone 12 g, Bacto yeast extract 24 g, glycerol 4 mL, KH_2PO_4 2.31 g, K_2HPO_4 12.54 g per 1 liter) at a ratio of 1% and incubated at 37 °C. In the case of P450 expression, 80 mg/L of 5-amino levulinic acid and 100 μM of $Fe(NH_4)(SO_4)_2$ was added in the TB broth medium, to facilitate heme biosynthesis [25]. The antibiotic spectinomycin (100 mg/L), carbenicillin (100 mg/L), and kanamycin (50 mg/L) were used to maintain plasmid. When the optical density of the culture at 600 nm reached 0.8, IPTG was added at a final concentration of 50 μM and incubated at 20 °C for 16 hours.

After induction, the cells were harvested by centrifugation and resuspended in 50 mM of potassium phosphate buffer (pH 7.2) containing 5% (v/v) glycerol. The cell suspension was diluted to make OD_{600} = 20 in final concentration, mixed with the substrate, and the biocatalysis reactions were performed at 14 °C for 16 hours or otherwise indicated. The reaction was carried out in GC vial or headspace vial with tightly closed lids. At substrate addition, vial and its contents were cooled by ice to reduce the volatilization of the substrate, especially (−)-limonene.

Analytical method

After the conversion reaction, vials were cooled by ice, then ethyl-acetate extraction was conducted. *E. coli* BL21(DE3) cells with a plasmid containing no insert were included as a negative control in these experiments. Obtained extracts were analyzed by gas chromatography (GC). GC-FID analysis was performed on a GC-2010Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (at 300 °C) and DB-1 column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA). The analysis was carried out with a temperature program of initial temperature 65 °C for 5 min, 5 °C/min to 145 °C, 25 °C/min to 250 °C, then hold at

300 °C for 3 min. The carrier gas was helium (120.7 kPa, 19.6 mL/min). Injection condition was split flow 1:10, 250 °C, linear velocity 35.0 cm/second.

GC mass spectrometry (GC-MS) analysis was performed on a GCMS-QP2010 system (Shimadzu) using an Rt-βDEX column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, RESTEK, Bellefonte, PA, USA). The analysis was carried out with a temperature program of initial temperature 50 °C for 5 min, 5 °C/min to 230 °C. The carrier gas was helium (0.7 mL/min). Injection condition was split flow 1:10, 220 °C, linear velocity 30.4 cm/second. The interface temperature was 220 °C. The detector worked in the scan mode and detection was performed in the range of m/z 40–400.

QconCAT standard protein preparation

Two tryptic peptides were chosen based on the preliminary study to represent each protein. The peptide sequences were concatenated in two different orders to make QconCAT1 and QconCAT2 (Fig. 5A). This artificial gene was synthesized and cloned into BamHI-XhoI site of the pET-28a expression vector by GenScript Biotech Corporation (Pis-cataway, NJ, USA). Internal BamHI and XhoI sites were removed by substitution with a synonymous codon (Additional file 3). Resulting QconCAT expression plasmids were transformed to *E. coli* BL21(DE3) and maintained in LB medium containing kanamycin (50 mg/L). A single colony was inoculated to 2 mL of the ^{13}C-M9 medium (Na_2HPO_4 6.78 g, KH_2PO_4 3 g, NH_4Cl 1 g, NaCl 0.5 g, $MgSO_4$ 0.24 g, $CaCl_2$ 11 mg, thiamine 10 mg, and [U-$^{13}C_6$] glucose 10 g per 1 liter) and incubated overnight at 37 °C. This seed culture was inoculated into 40 mL of the ^{13}C-M9 medium at a ratio of 1% and incubated at 37 °C. When the optical density of the culture at 600 nm reached 0.6, IPTG was added at a final concentration of 1 mM and incubated at 30 °C for 46 hours. Cells were harvested by centrifugation and resuspended in 10 mL of xTractor buffer (Clontech Laboratories, Inc., Mountain View, CA, USA) and the crude lysate was obtained according to the manufacturer's protocol for extracting proteins from bacterial cell culture. The lysate was His-tag purified using Capturem™ Maxiprep Kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol. Purified protein concentration was measured by the Bradford method. Sample purity was confirmed by SDS-PAGE.

Proteome analysis

Total proteins were extracted as described previously with minor modification as following [26]. *E. coli* cells were harvested by centrifugation such that the OD_{600} × volume (mL) = 50, washed with M9 medium once, and then frozen at − 80 °C until analysis. Cell pellets were resuspended in 1 mL lysis buffer (50 mM HEPES at pH 7.5, 5% (v/v) glycerol, 15 mM dithiothreitol, 100 mM KCl, and 5 mM EDTA). Resuspended cells were disrupted using a multi-beads shocker (Yasui Kikai Corporation, Osaka, Japan) with glass beads YGB01 (diameter 0.1 mm, Yasui Kikai Corporation) at 10 cycles of 2500 rpm for 30 seconds with a 30 seconds interval, and then centrifuged to collect the supernatant. The supernatants were used for protein quantitation using the Bradford method.

Next, 50 μg of total protein and 2 μg of QconCAT protein was supplemented with denaturing buffer (500 mM Tris-HCl at pH 8.5, 10 mM EDTA, 7 M Guanidine HCl) to the total volume of 220 μL. One

microliter of 50 mg/mL dithiothreitol was added and mixed by vortexing at room temperature for 1 h. Then, proteins were alkylated with 2.5 mL of 50 mg/mL iodoacetamide (IAA) with vortex mixing in the dark at room temperature for 1 h. Six hundred microliters of ice-cold methanol, 150 µL of chloroform, and 450 µL of cold water were consecutively added to the lysates and mixed gently after the addition of each component. After centrifugation at 20,000 × g for 5 min at 4 °C, the upper phase was discarded. Subsequently, 450 µL of methanol was added to the bottom phase as well as the interphase, and proteins were precipitated by centrifugation under the same conditions. Trypsin/LysC digestion was performed as described previously [27]. Proteins were dissolved in 9 µL of 6 M urea for 10 min by vortex mixing. Then, 36 µL of 0.1 M Tris-HCl (pH 8.5) was added to the protein solution and mixed via sonication. Proteolytic digestion into peptides was performed using 1 µL of 0.5 mg/mL lysyl endopeptidase (Lys-C; Wako Pure Chemical Industries, Osaka, Japan) at a final concentration of 1% (w/w) Lys-C per sample protein and 2.5 µL of 1% w/v ProteaseMax Surfactant Trypsin Enhancer (Promega, Madison, WI, USA) at 25 °C for 3 h, followed by 1 µL of 0.5 mg/mL L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Promega) at a final concentration of 1% (w/w) trypsin per sample protein at 37 °C for 16 h. Following trypsin digestion, 7.5 µL water and 3 mL of 50% (v/v) formic acid were added to the protein sample, which was then centrifuged at 20,000 × g for 5 min. Finally, 12 µL of the sample was mixed with 36 µL of 5% formic acid, and the mixtures were desalted with C18-StageTips [28–30].

Samples were analyzed by nano-liquid chromatography mass spectrometry (nano-LC-MS/MS). The nano-LC-MS/MS system comprised an LC-20Adnano and an LCMS-8060 triple-quadrupole mass spectrometer with an electrospray ionization ion source (Shimadzu). Sample separation was done by nano-LC (LC-20Adnano), and electrospray ionization was performed by LCMS-8060. All analytical methods used were performed as described previously [31–33]. The multiple reaction monitoring (MRM) method used to relatively quantify 5 proteins was created by the open software Skyline version 4.1 [34], and shown in additional file 4. Peptides were relatively quantified by the peak area ratio of the ^{12}C sample to the ^{13}C sample derived from QconCAT1 protein.

Abbreviations

AQUA, absolute quantification; CAGR, compound average growth rate; CDH, carveol dehydrogenase; CPR, cytochrome P450 reductase; EDTA, ethylenediaminetetraacetic acid; DCPIP, dichlorophenolindophenol; FID, flame ionization detector; GC, gas chromatography; GC-MS, gas chromatography mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAA, iodoacetamide; IPTG, isopropyl β-D-1-thiogalactopyranoside; ISPD, carveol dehydrogenase from peppermint; MCS, multiple cloning site; MRM, multiple reaction monitoring; MS, mass spectrometry; OD, optical density; PSAQ, protein standard absolute quantification; QconCAT, quantification concatemer; SDS-PAGE. sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

The authors provide consent for publication.

Availability of data and materials

The data supporting the conclusions of this article are included with the article. Strains examined are available from the corresponding author.

Competing interests

The authors declare no competing interests.

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Authors' contribution

Conceived and designed the experiments: MS and YN. Performed the experiments: EY, MK, and MS. Performed the proteome analysis: FM. Wrote the paper: EY. Edited the paper: MK, MS, FM, AO, YN, YU, and JI. Project administration: MS, AO, and YN. Supervised the whole work: AO, YN, YU, and JI. Funding acquisition: AK. All authors read and approved the final manuscript.

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Figures

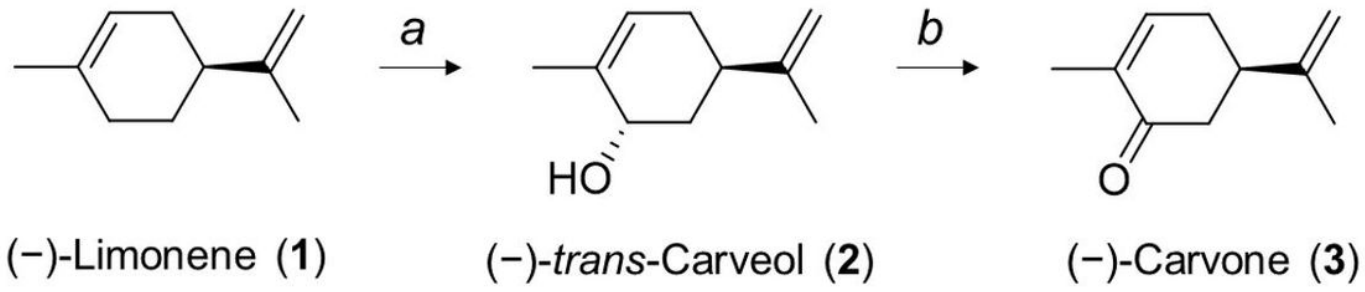

Figure 1

Biosynthesis pathway of carvone from limonene in Mentha sp. Pathway for the biosynthesis of (−)-carvone from the primary precursor (−)-limonene is shown. The indicated enzymes are (a) cytochrome P450 limonene-6-hydroxylase, and (b) carveol dehydrogenase.

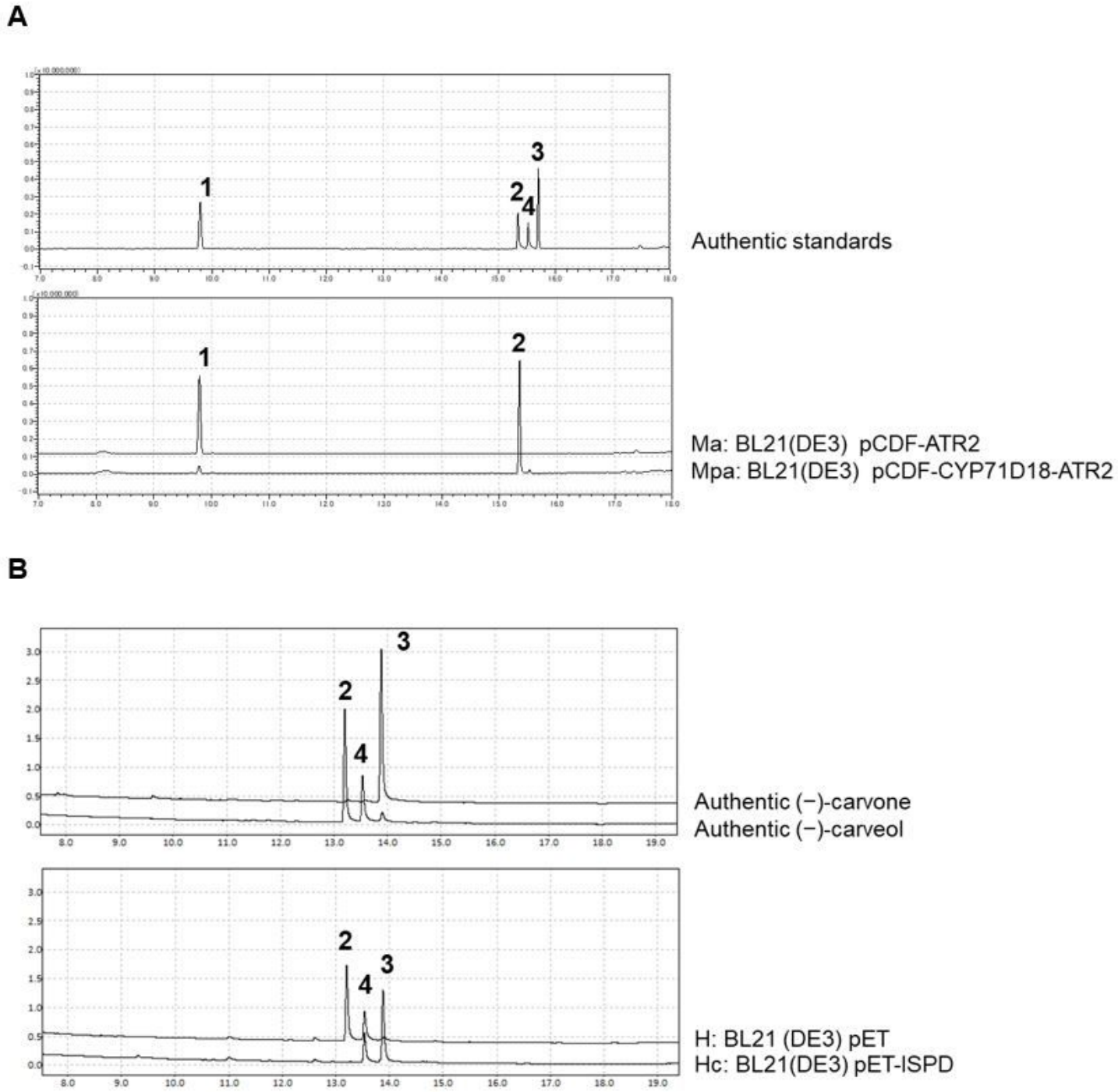

Figure 2

Limonene and carveol conversions for tests of constructed recombinant E. coli strains using a whole-cell biocatalytic system. (A) Reaction with (−)-limonene and cells expressing cytochrome P450 limonene-6-hydroxylase gene from spearmint (CYP71D18) and/or cytochrome P450 reductase (CPR) gene from A. thaliana (ATR2) (Mpa and Ma strains; BL21(DE3) pCDF-CYP71D18-ATR2 and BL21(DE3) pCDF-ATR2). Mpa strain specifically converted (−)-limonene to (−)-trans-carveol. (B) Reaction with (−)-carveol and cells expressing carveol dehydrogenase (CDH) gene from peppermint (ISPD) (Hc strain; BL21(DE3) pET-ISPD). Hc strain specifically converted (−)-trans-carveol to (−)-carvone. Enzymatic activities were

confirmed by gas chromatography (GC) analysis. Upper and lower panels indicate the authentic standards and test samples; (−)-limonene (1), (−)-carveol (2, 4), and (−)-carvone (3). Commercially available (−)-carveol contains (−)-trans-carveol (2), (−)-cis-carveol (4), and trace of (−)-carvone (3).

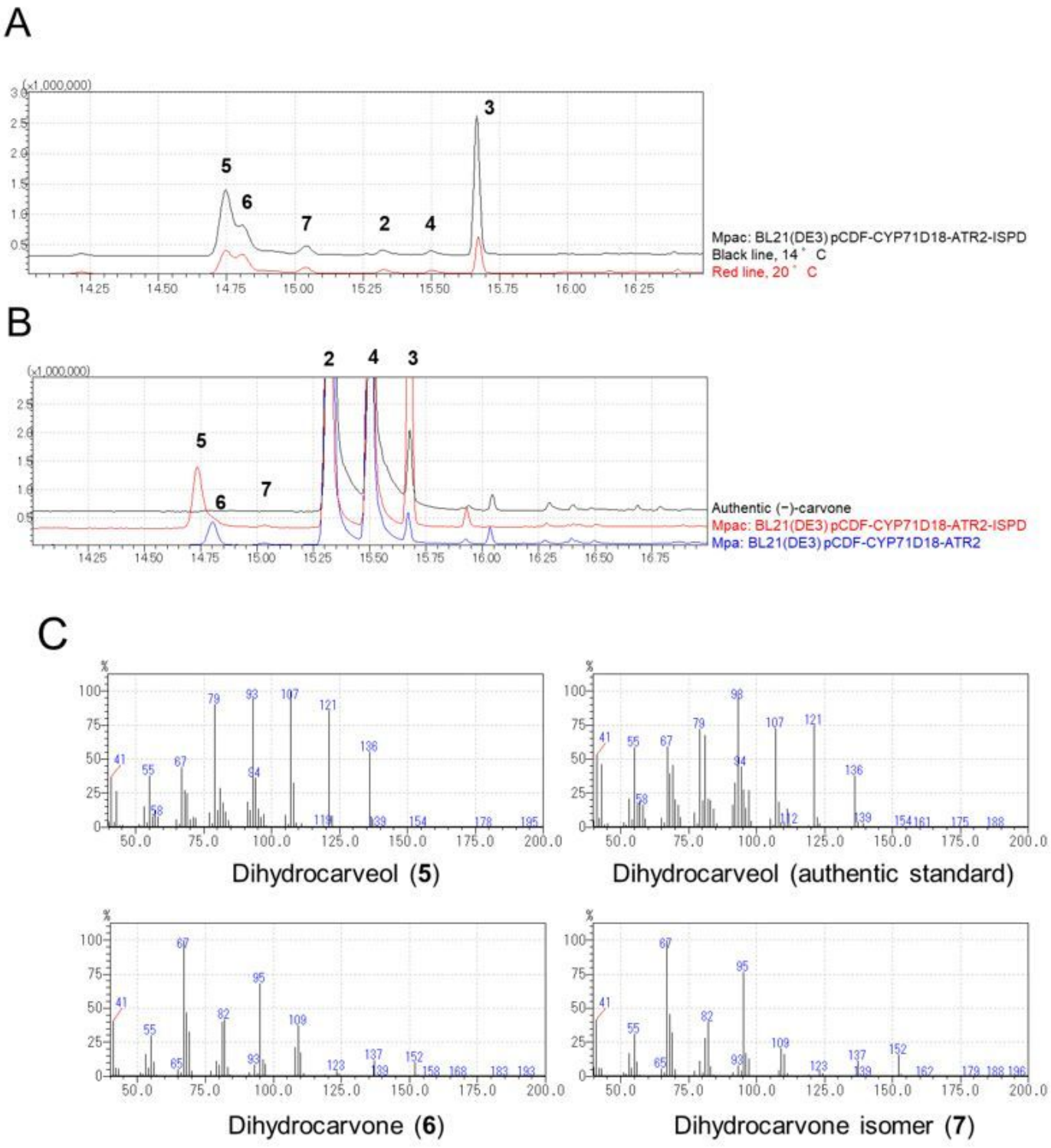

Figure 3

Carvone production from limonene using a recombinant E. coli strain co-expressing P450, CPR and CDH, and identification of by-product compounds. Cytochrome P450 limonene-6-hydroxylase gene from

spearmint (CYP71D18), cytochrome P450 reductase (CPR) gene from A. thaliana (ATR2), and carveol dehydrogenase (CDR) gene from peppermint (ISPD) were used as P450, CPR, and CDH, respectively. Their enzymatic activities were confirmed by gas chromatography (GC) analysis. (A) Reaction with (−)-limonene and cells co-expressing P450, CPR and CDH (Mpac strain; BL21(DE3) pCDF-CYP71D18-ATR2-ISPD) under different temperature conditions (black line, 14 °C; and red line, 20 °C). Mpac strain converted (−)-limonene to (−)-carvone but also generated by-products. (B) Reaction with (−)-carveol and cells expressing P450, CPR, and CDH (Mpac strain, red line), and cells expressing P450 and CPR (Mpa strain, blue line), respectively. Mpac strain generated an undetermined by-product, compound (5). Mpa strain generated an undetermined by-product, compound (6, 7). Black line indicates authentic (−)-carveol standard. Commercially available (−)-carveol contains (−)-trans-carveol (2), (−)-cis-carveol (4), and trace of (−)-carvone (3). (C) Mass spectrometry (MS) fragment pattern of the reaction products with (−)-limonene and cells expressing P450, CPR and CDH (Mpac strain), and the authentic standard (dihydrocarveol). Fragment patterns determined by GC-MS analysis indicated that the undetermined by-products were dihydrocarveol and dihydrocarvone. Numbers indicate the following compound: (−)-trans-carveol (2), (−)-carvone (3), (−)-cis-carveol (4), dihydrocarveol (5), dihydrocarvone (6), dihydrocarvone isomer (7).

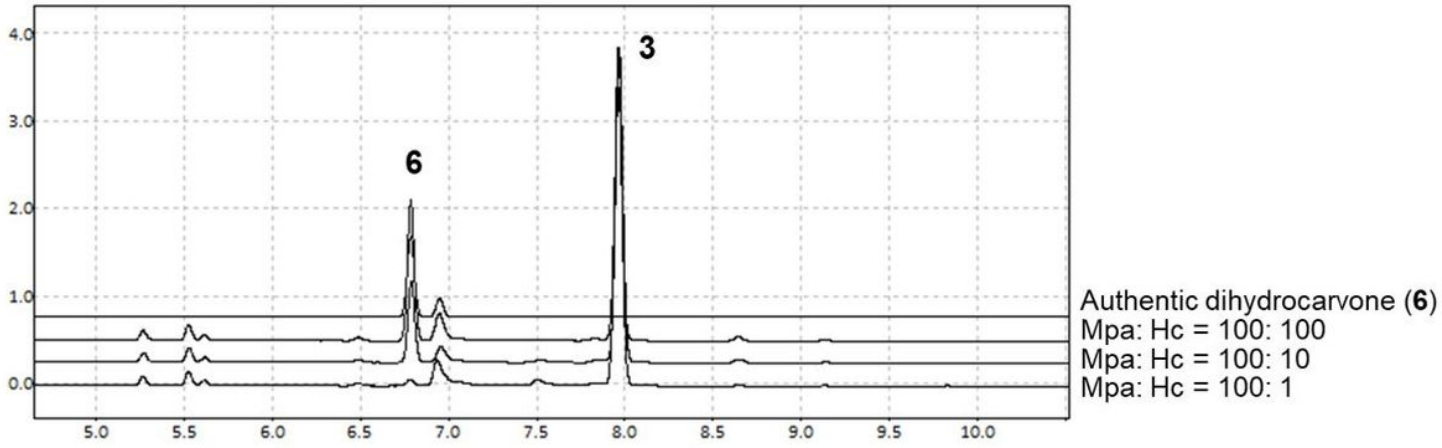

Figure 4

Optimization of enzyme balance by mixing two strains expressing P450/CPR and CDH enzymes. Strains expressing P450 and CPR (Mpa strain; BL21(DE3) pCDF-CYP71D18-ATR2) or CDH (Hc strain; BL21(DE3) pET-ISPD) were mixed in various ratios to find the optimum balance for converting (−)-limonene to (−)-carvone. Varied P450/CDH input results in different (−)-carvone (3) production along with by-product dihydrocarvone (6) production.

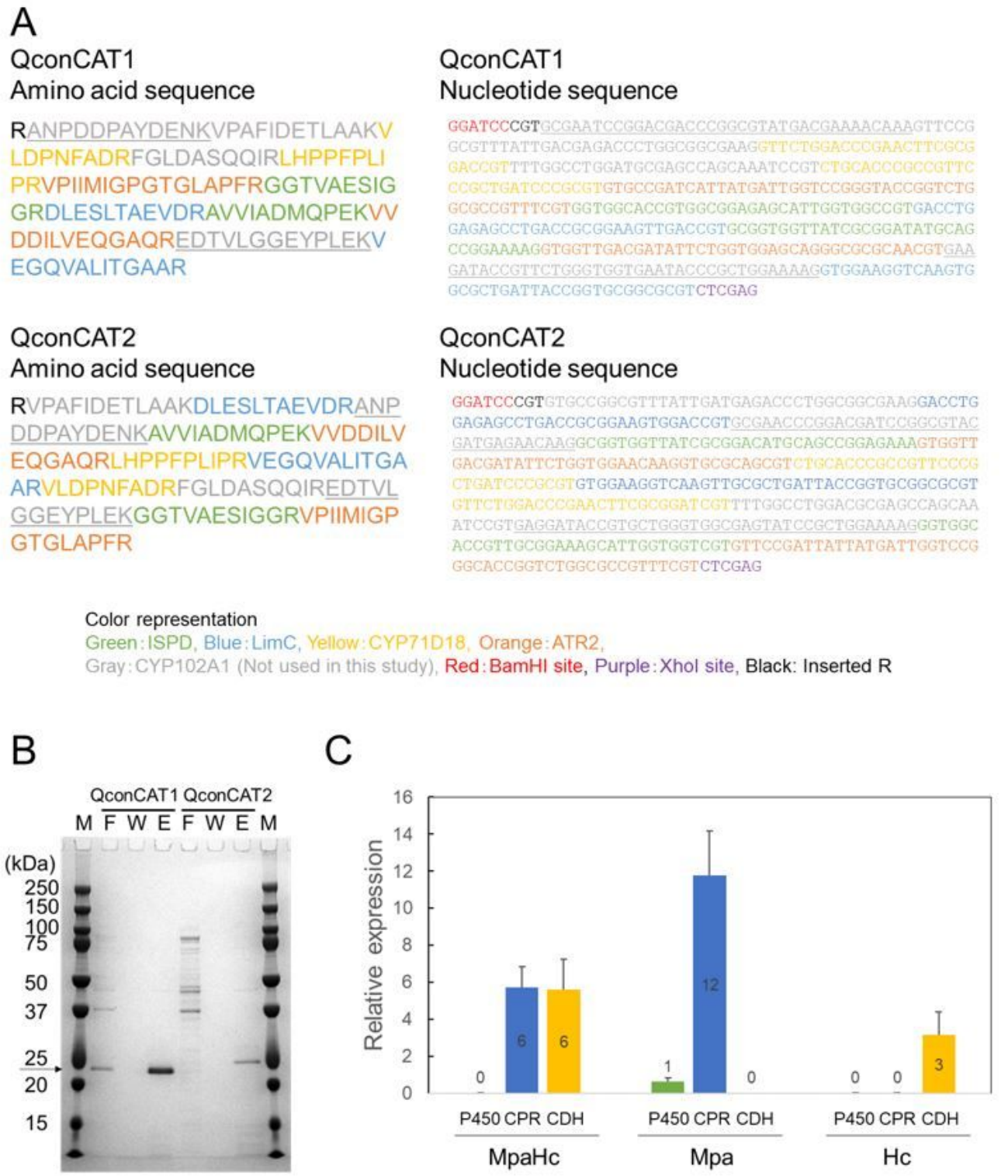

Figure 5

Enzyme expression level quantification by proteome analysis using QconCAT method. To find the optimum balance for converting limonene to carvone, the semi-quantitative proteome analysis using QconCAT protein was conducted for strains expressing P450, CPR or CDH. (A) QconCAT standard protein design. Two tryptic peptides were chosen based on the preliminary study to represent each protein. The peptide sequences were concatenated in two different orders to make QconCAT1 and QconCAT2. Internal

BamHI and XhoI sites were substituted with synonymous codons. Codons were optimized for E. coli expression by GenScript. (B) Purification of QconCAT proteins. SDS-PAGE image showed that QconCAT proteins (20 kDa, indicated by an arrow) were correctly expressed and purified. Applied samples per lane were indicated as following; M: molecular mass standard marker, F: flow through, W: wash, E: elution fractions. The positions of the molecular mass standards are indicated. (C) Semi-quantitative proteome analysis using QconCAT method. Relative expression amounts of P450, CPR and CDH in each strain were determined using QconCAT1 protein as a standard. MpaHc strain expressed P450, CPR and CDH (BL21(DE3) pCDF-CYP71D18-ATR2, pET-ISPD). Mpa strain expressed P450 and CPR (BL21(DE3) pCDF-CYP71D18-ATR2). Hc strain expressed CDH (BL21(DE3) pET-ISPD). The relative expression amount was shown as an average of three independent clones. Error bars represent standard deviations of n = 3.

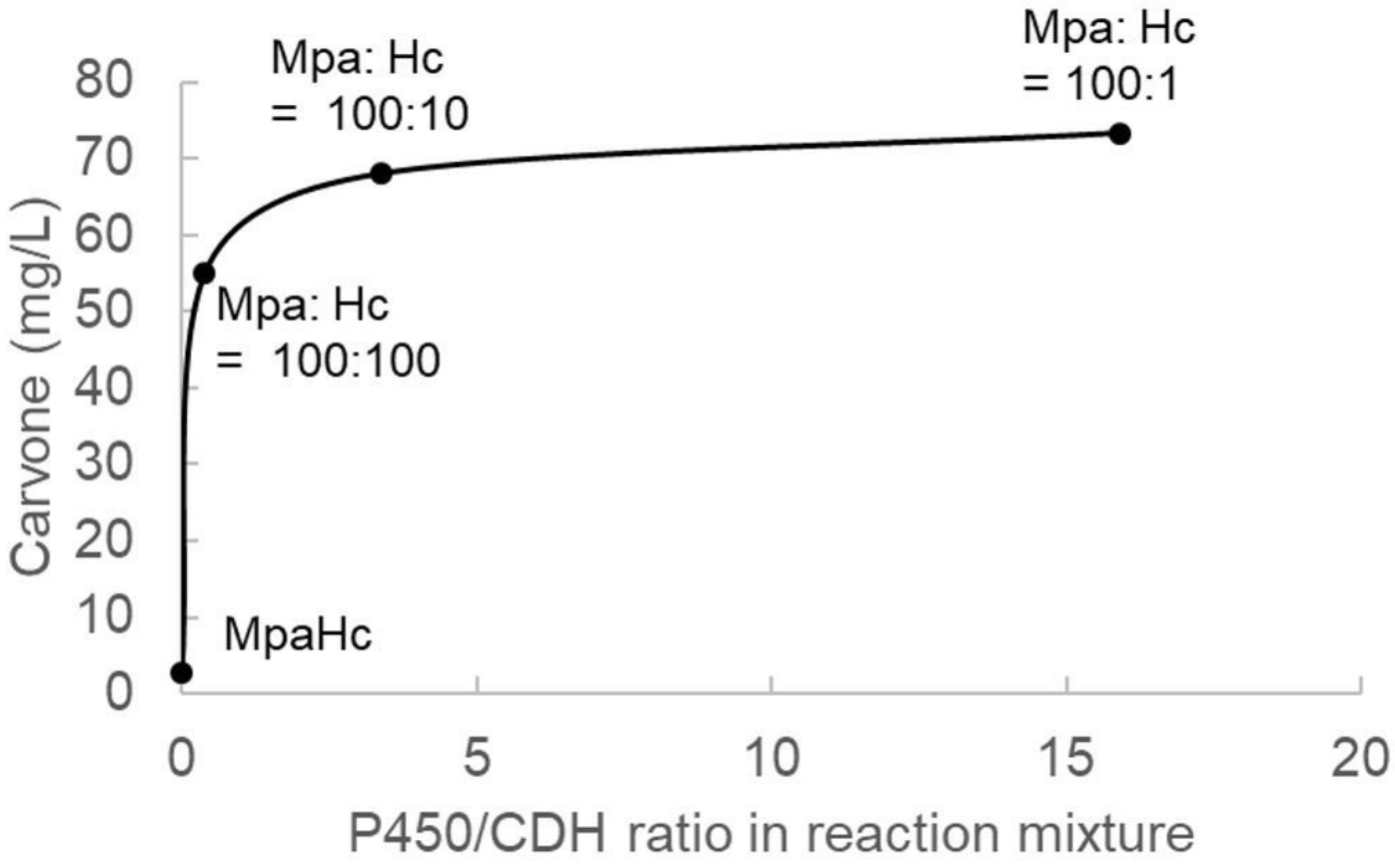

Figure 6

Correlation of P450/CDH protein ratio and produced carvone amount. P450/CDH ratio was calculated based on the QconCAT proteome analysis and its correlation between carvone production was shown. Each plot on the graph represents 4 different conditions. From left to right MpaHc (BL21(DE3) pCDF-CYP71D18-ATR2, pET-ISPD) showing P450/CDH ratio of 0.004, Mpa (BL21(DE3) pCDF-CYP71D18-ATR2) and Hc (BL21(DE3) pET-ISPD) mixture (100:100) showing 450/CDH ratio of 0.388, Mpa and Hc mixture (100:10) showing 450/CDH ratio of 3.4, and Mpa and Hc mixture (100:1) showing P450/CDH ratio of 16 respectfully. Mpa and Hc mixture ratio is based on the OD value of each strain.

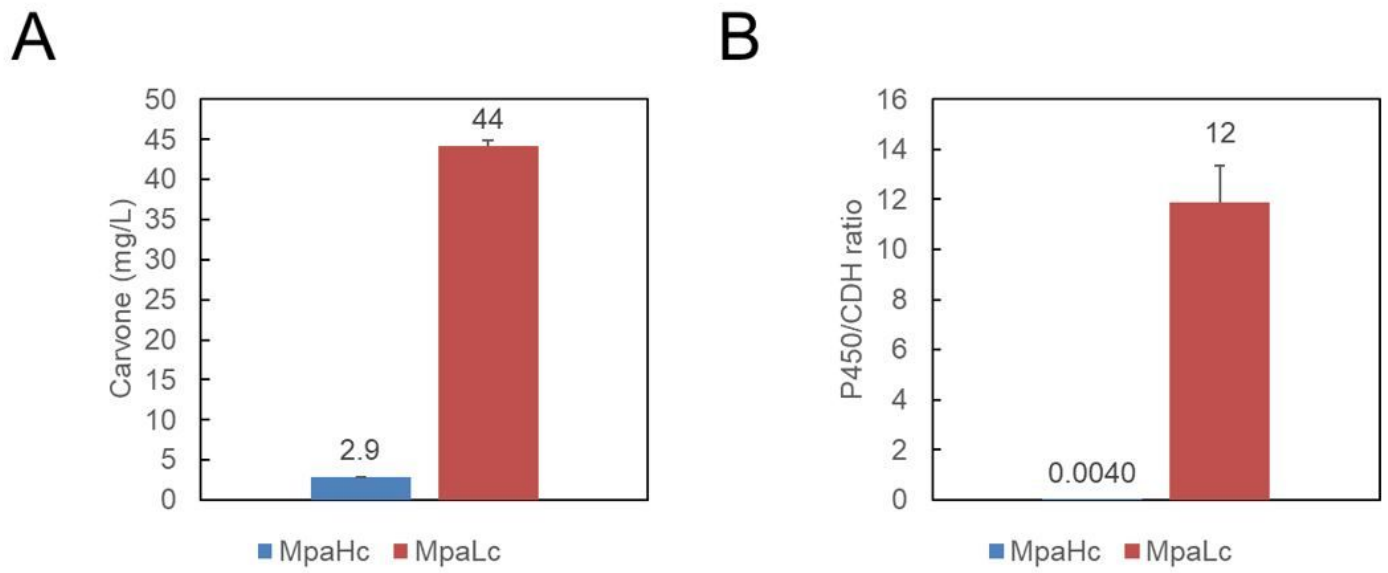

Figure 7

Carvone conversion by single cell reaction. (A) The novel strain MpaLc (BL21(DE3) pCDF-CYP71D18-ATR2, pMW-lSPD) along with previously constructed strain MpaHc (BL21(DE3) pCDF-CYP71D18-ATR2, pET-lSPD) were analyzed using carvone biocatalysis assay with 100 mg/L (−)-limonene as a starting substrate. Error bars represent standard deviations of n = 3. (B) P450/CDH ratio was calculated based on the QconCAT proteome analysis of MpaHc and MpaLc strains. Error bars represent standard deviations of n = 3.

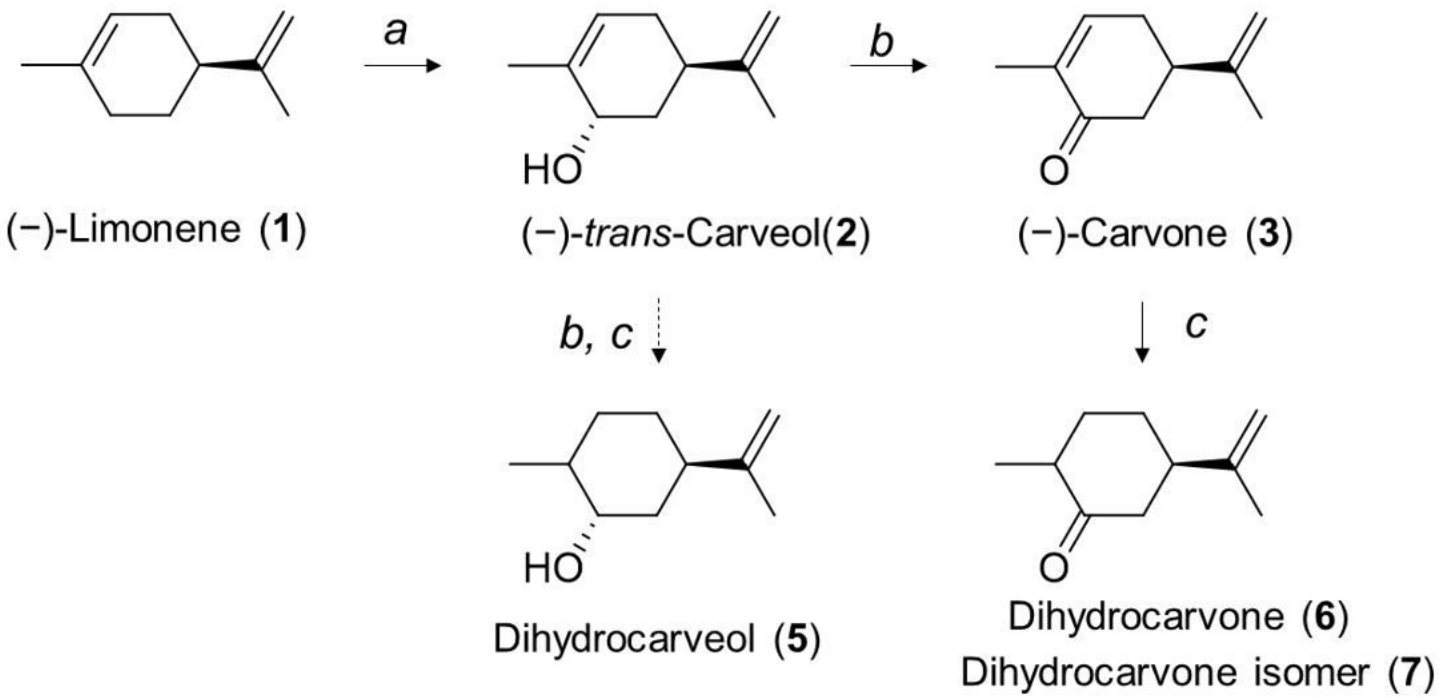

Figure 8

Hypothetical by-product formation pathway. Pathway for the biosynthesis of (−)-carvone from the primary precursor (−)-limonene is shown. The indicated enzymes are (a) cytochrome P450 limonene-6-hydroxylase, (b) carveol dehydrogenase, and (c) unknown endogenous enzyme.

Supplementary Files

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